Heat Shock Protein 70 Expression in Silver Sea Bream (*Sparus sarba*) Tissues: Effects of Hormones and Salinity

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Abstract

In this thesis, the HSP70 expression in whole blood of *Sparus sarba* was studied. In the first and second parts, the HSP70 expression in whole blood in response to *in vitro* thermal stress and hormone treatments was investigated. In the third part, fish were subjected to *in vivo* osmotic stress and the blood HSP70 expression was then investigated. The HSP70 expression in the whole present study was assessed by indirect ELISA. Since the standard curve of bovine HSP70 and the titration curve of serially diluted whole blood protein displayed parallelism, the indirect ELISA for HSP70 as developed in the present study provided a valid quantification of HSP70 in whole blood.

The HSP70 levels of whole blood of *Sparus sarba* were upregulated after exposure to thermal stress [cold shock (12°C), heat shock (27°C) and severe heat shock (32°C)] for 2 hours *in vitro*. The temperature at which HSP70 synthesis was induced was between 19 and 27°C and the maximal HSP70 induction was at 32°C. The levels of HSP70 in whole blood also significantly increased at 12°C from a control temperature of 19°C. The results supported the notion that both heat and cold stress were potent stimuli for blood HSP70 upregulation in fish.

Since the endocrine regulation of HSP70 expression in fish is not clear, one of the aims of the present study was to establish whether key hormones directly regulate blood HSP70 expression in *Sparus sarba*. *In vitro* exposure to cortisol, the classic stress hormone, was found to have no effect on the HSP70 levels in whole blood. The result supports the contention that a definite relationship between cortisol and

blood HSP70 expression in fish cannot be established. Recombinant bream growth hormone (rbGH), insulin-like growth factor-I (rbIGF-I) and ovine prolactin (oPRL) all significantly reduced HSP70 levels in whole blood in a dose-dependent manner. Since HSP70 expression indicates the stress levels, the results supported the notion that growth hormone, IGF-I and prolactin might be related to a reduced stress state and have a protective effect on against stress.

Lastly, the effect of 4-week salinity adaptation on HSP70 expression in whole blood of euryhaline teleost *Sparus sarba* was examined in the study. This is an *in vivo* experiment. The lowest blood HSP70 expression was found in fish adapted to an iso-osmotic salinity of 12 ppt. The HSP70 levels were highest in 50 ppt and 6 ppt environments. By using HSP70 expression as a bioindicator of stress, the results indicated that iso-osmotic salinity would bring about the least stress level while 50 ppt and 6 ppt were the most stressful salinities to *Sparus sarba*.

摘要

這論文討論了鯛魚(*Sparus sarba*)血液樣本熱休克蛋白 70 的表達。第一和第二部份的研究是體外實驗，正常鯛魚的血液樣本分別接受不同的溫度與激素處理。第三部份的研究是體內實驗，收集鯛魚適應於不同鹽度的血液樣本。熱休克蛋白 70 之數值用間接酶聯免疫法檢測。由於以連續稀釋的牛熱休克蛋白 70 和鯛魚血蛋白繪製的標準曲線和滴定曲線有平行性，所以改良的間接酶聯免疫法能有效檢測鯛魚血液樣本熱休克蛋白 70 的數值。

在第一個體外實驗裏，血液樣本持續二小時接受三個溫度刺激。三個溫度刺激包括冷激(12°C)、熱激(27°C)和超熱激(32°C)。結果顯示冷激、熱激和超熱激都能明顯地上調鯛魚血液熱休克蛋白 70 的水平，其中超熱激最能誘導熱休克蛋白 70 的合成。結果說明了熱激和冷激能有效促進魚類血液裏的熱休克蛋白 70 的表達。

第二部份的實驗目的是研究激素在體外對魚類血熱休克蛋白 70 表達的作用。結果顯示皮質醇對血熱休克蛋白 70 的表達沒有明顯的影響，而重組鯛生長激素(rbGH)、重組鯛類胰島素生長因子-I(rbIGF-I)和牛催乳激素(oPRL)都能以劑量依賴方式顯著地降低熱休克蛋白 70 的表達。由此推斷皮質醇和魚類熱休克蛋白 70 的表達沒有明確的關係，而生長激素、類胰島素生長因子-I 和催乳激素都可能與降低脅迫有關，因而可以保護魚類面對外界刺激。

第三部份的研究針對廣鹽性的鯛魚血液熱休克蛋白 70 的表達在不同鹽度下的變化。首先，鯛魚適應於五個不同的鹽度，四星期後，血液樣本的熱休克蛋白 70 以間接酶聯免疫法檢測。五個鹽度包括高滲鹽度(50 ppt)、正常海水鹽度(33

ppt)、等滲鹽度(12 ppt)、低滲鹽度(6 ppt)和淡水鹽度(0 ppt)。結果顯示熱休克蛋白70 在等滲鹽度(12 ppt)環境下的表達最低，而在 6 ppt 和 50 ppt 的表達最高。結果表示魚類在 12 ppt 等滲的環境下所受脅迫最低。

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List of abbreviations

ACTH	adrenocorticotropic hormone / corticotropin
ADP	adenosine diphosphate
AR	androgen receptor
ATP	adenosine triphosphate
Bip	immunoglobulin heavy chain binding protein
BKD	bacterial kidney disease
BKME	bleached kraft pulp mill effluent
BNF	β-naphthoflavone
BSA	bovine serum albumin
COOH-terminus	carboxy-terminus
cpn10	chaperonin 10
cpn60	chaperonin 60
ct-hsp70	cytosolic HSP70
CTR	carboxy-terminal repeat
DMEM	Dulbecco's Modified Eagle Medium
DR	dioxin receptor
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-protein ligase (recognin)
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum ; estrogen receptor
FGF	fibroblast growth factor
GH	growth hormone / somatotropin
GH-R	growth hormone receptor
GRF / GHRH	growth hormone-releasing factor (hormone)
GR	glucocorticoid receptor
Grp75	75-kDa glucose regulated protein
Grp78	78-kDa glucose regulated protein
Grp94	94-kDa glucose-regulated protein

GTH	gonadotropin hormone
Hip	HSP70 interacting protein
Hop	HSP90-HSP70 organizing protein
HPA axis	hypothalamic-pituitary-adrenal axis
HPI axis	hypothalamo-pituitary-interrenal axis
Hsc70	70-kDa heat shock cognate
HSE	heat shock element
HSF	heat shock transcription factor
HSP	heat shock protein
HSP10	10-kDa heat shock protein
HSP30	30-kDa heat shock protein
HSP60	60-kDa heat shock protein
HSP70	70-kDa heat shock protein
HSP90	90-kDa heat shock protein
HSP100	100-kDa heat shock protein
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IL-1	interleukin-1
IP	immunophilin
JAK	Janus kinase
kD	kilodalton
LMW	low-molecular-weight
LZ	leucine zipper
MAPKAP	mitogen-activate protein kinase-activated protein
MR	mineralocorticoid receptor
MSH	melanocyte-stimulating hormone
mRNA	messenger RNA
MSL	Marine Science Laboratory
mt-hsp70	mitochondrial HSP70
NH_2-terminus	amino-terminus
oPRL	ovine prolactin
PAS(+) / PIPAS	cells in pars intermedia that can be stained with periodic

cells	acid-Schiff (PAS) reagent
PAS reagent	periodic acid-Schiff reagent
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PL	placental lactogen / somatomammotropin
POMC	proopiomelanocortin
PPIase	peptidylprolyl isomerase
ppt	part per thousand
PRL	prolactin
PRL-R	prolactin receptor
prp73	73-kDa peptide recognition protein
PR	progesterone receptor
RBC	red blood cell
rbGH	recombinant bream growth hormone
rbIGF-I	recombinant bream insulin-like growth factor-I
SEM	standard error
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
sHSP	small heat shock protein
SL	somatolactin
SM	somatomedin
SRIF / SRIH	somatostatin / somatotropin release-inhibiting factor (hormone)
sst	somatostatin receptor
STAT	signal transducers and activators of transcription
TNF-	tumor necrosis factor
Ub	ubiquitin

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General introduction

Heat shock proteins (HSPs) are stress proteins and also molecular chaperones. There are six major families of heat shock proteins and HSP70 has been most widely studied. Since HSP70 synthesis can be induced by many proteotoxic stresses and these proteins are highly conserved, HSP70 can serve as a useful molecular biomarker of environmental stress (Ryan and Hightower, 1996). Therefore, the stress levels of fish can be determined by using HSP70 expression as a bioindicator of stress.

In this study, HSP70 expression in whole blood of silver sea bream or gold-line sea bream (*Sparus sarba*) was examined. There are several advantages in choosing whole blood as an *in vitro* system to study HSP70 expression. First of all, blood is easily collected and experimentally manipulated. Likewise, collecting blood is a non-terminal means for assessing the impact of environmental stresses on fish. And furthermore the main component of whole blood is red blood cells (RBCs). Unlike enucleated mammalian red blood cells, the fish RBCs are nucleated and are capable of protein synthesis, thus providing a useful model for studying HSP70 expression *in vitro* (Currie and Tufts, 1997).

As the term "heat shock proteins" suggests, synthesis of HSPs is inducible by elevated temperatures. Therefore, the first objective of this study was to establish whether fish blood HSP70 expression could be used as a bioindicator of thermal stress. In this *in vitro* experiment, whole blood of *Sparus sarba* was exposed to four different thermal shocks: heat shock (27°C), severe heat shock (32°C), cold shock (12°C) and the control temperature of 19°C. In order to quantify the blood HSP70 levels in silver

sea bream, an indirect ELISA was developed.

On the other hand, the hormonal regulation of HSP expression has not been adequately addressed for fish. Therefore, another aim of this study was to establish whether key hormones directly regulate whole blood HSP70 expression in *Sparus sarba*. In this study, four hormones were chosen for the investigation of their effects on HSP70 expression. They were cortisol, recombinant bream growth hormone (rbGH), ovine prolactin (oPRL) and recombinant bream insulin-like growth factor-I (rbIGF-I). Cortisol is the major corticosteroid in the majority of teleost fish species. It is a 21-C steroid produced by the interrenal tissue and is a classic stress hormone. Stressors, either acute or chronic, increase the plasma cortisol levels by activation of the hypothalamo-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997). However, daily administration of exogenous cortisol was found to have no effect on hepatic HSP70 expression in *Sparus sarba* (Deane *et al.*, 1999). Growth hormone is so called because of its growth-promoting effect. Growth hormone and prolactin are adenohypophysial tropic hormones and also peptides that belong to the same family. IGF-I is structurally related to insulin but it is a mediator of the growth-promoting effects of growth hormone. Although stress-induced growth suppression was suggested, the importance of growth hormone, prolactin and IGF-I during stress has not yet been established in fish. Downregulation of hepatic HSP70 expression was observed in *Sparus sarba* receiving daily intraperitoneal injections of rbGH and oPRL (Deane *et al.*, 1999). The results suggested that growth hormone and prolactin might have a protective effect on stress tolerance in fish. Some works have examined the relationship between the hypothalamic-pituitary-adrenal (HPA) axis and HSP70 expression in mammals. The adrenal cortex of mammals is homologous to the

interrenal tissue of teleosts. Exogenous administration of ACTH was shown to restore the rapid induction of HSP70 mRNA expression in the adrenal cortex of the hypophysectomized rats in response to restraint stress (Blake *et al.*, 1991). The results from another study showed that both HSP70 mRNA expression in the adrenal cortical tissue and plasma ACTH levels of rats undergoing restraint stress was attenuated by the synthetic glucocorticoid dexamethasone (Udelsman *et al.*, 1994).

Lastly, the effect of salinity stress on blood HSP70 expression in euryhaline teleost *Sparus sarba* was addressed. In this *in vivo* experiment, silver sea bream was acclimated to various salinities (50 ppt, 33 ppt, 12 ppt, 6 ppt and 0 ppt) for 1 month before blood sampling. 50 ppt is a hyper-osmotic salinity; 33ppt is the salinity of normal seawater; 12 ppt is the iso-osmotic salinity of silver sea bream; 6 ppt is a hypo-osmotic salinity and 0 ppt is the salinity of fresh water. Together with *Chrysophyrs major* (red sea bream) and *Mylio macrocephalus* (black sea bream), *Sparus sarba* is a member of the Sparidae and carnivorous. *Sparus sarba* is marine food fish that are found in the temperate and subtropical waters of western Pacific Rim. Silver sea bream has been adapted well in a hypo-osmotic environment of 6 ppt by Kelly and Woo (1999). During the hypo-osmotic adaptation, no significant change in the serum levels of Na^+ and Cl^- and muscle moisture content were observed. In this study, the number of branchial chloride cells was reported to increase after 21 days of hypo-osmotic acclimation. However, the increase in the number of chloride cells in *Sparus sarba* probably did not result from hypercortisolemia as the hypo-osmotic adaptation was also shown to have no effect on serum cortisol levels (Kelly and Woo, 1999). Iso-osmotic adaptation has been reported to enhance fish growth and stimulate the non-specific immune response of fish (Narnaware *et al.*, 2000).

Iso-osmotic environment is therefore believed to be an environment with least stress level which is conducive for introduction to fish culture practice. The objective of this study was to test the hypothesis that iso-osmotic environment is an environment of minimal stress by using HSP70 expression as a bioindicator of stress.

Chapter 1:

Literature review

Chapter 1: Literature review

1.1. Heat shock proteins (HSPs)

1.1.1. Introduction

Heat shock proteins (HSPs) are a group of intracellular proteins of which synthesis is induced by exposure to heat or other stresses. Also, many of these proteins are constitutively expressed in normal unstressed cells. The functions of HSPs are important in all cells as the amino acid sequence are evolutionarily high conserved among diverse organisms from prokaryotes to man.

They are called "heat shock proteins" because the synthesis of this group of proteins is induced by elevated temperature. In 1962, F. Ritossa made the original finding and reported new salivary gland polytene chromosome puffs in fruit fly, *Drosophila busckii* after heat shock. Due to high intracellular protein concentration (100-150 mg/ml) and interactions of exposed hydrophobic amino acid surfaces, protein aggregation is a problem a cell must face. Under elevated temperatures, heat shock proteins prevent protein aggregation through binding to their hydrophobic surfaces exposed by denaturation (Kabakov and Gabai, 1997).

Heat shock proteins are also "stress proteins". In fact, synthesis of heat shock proteins can also be induced in response to many other proteotoxic stresses other than heat shock. These stresses can be either abiotic like thermal stress and chemical exposures, or biological such as pathogenic invasions (Iwama *et al.*, 1998).

Heat shock proteins are also referred to as "molecular chaperones" as they prevent protein aggregation and misfolding by assisting correct folding of nascent and newly synthesized proteins, directing assembly and disassembly of oligomeric structures, facilitating protein translocation across membranes, such as those of mitochondria, and refolding or degradation of denatured and abnormal proteins (Kabakov and Gabai, 1997).

Heat shock proteins are commonly classified on the basis of molecular weight in kilodaltons (kDa) and are divided into six families. They are HSP100, HSP90, HSP70, HSP60, small or low-molecular-weight heat shock proteins and ubiquitin (Kabakov and Gabai, 1997).

1.1.2. The various heat shock proteins

1.1.2.1. HSP100s

This family comprises highly conserved heat shock or stress proteins with molecular weight from 104 to 110 kDa in both prokaryotes and eukaryotes. Members of the family are all ATPases and usually contain two ATP-binding domains. The family is divided into three subfamilies based on the organization of these two highly conserved ATP-binding domains, namely ClpA, ClpB and ClpC (Gottesman *et al.*, 1990). However, HSP100 in *Escherichia coli* and mammals have one ATP-binding domain only (Gottesman *et al.*, 1990).

Hsp104 is a molecular chaperone required for thermotolerance in yeast against protein aggregation by high temperatures. It also protects yeast from high concentrations of ethanol but not from cadmium (Sanchez *et al.*, 1992).

Some members of this family are involved in proteolysis and degradation of stress-damaged proteins. The ATP-dependent Clp (Ti) protease in *Escherichia coli* has two components, ClpA and ClpP. ClpA is a hexameric ring and ClpP is composed of two heptameric rings. ClpA is a protein-activated ATPase and controls the proteolytic activity of ClpP (Hwang *et al.*, 1988). ClpA binds substrate proteins which should then be unfolded to allow translocation into the ClpP for degradation (Hoskins *et al.*, 2000).

1.1.2.2. HSP90s

HSP90 family include heat shock proteins of molecular weight from 82 to 94 kDa. They are highly conserved in all organisms. In eukaryotic cells, HSP90 is one of the most abundant proteins, making up 1–2 % of total cellular protein even under unstressed conditions (Scheibel and Buchner, 1998). HSP90 exists as a dimer, either as Hsp90α/α and Hsp90β/β homodimers or as an Hsp90α/β heterodimer (Perdew *et al.*, 1993).

In eukaryotes, there is a homologue of Hsp90 in endoplasmic reticulum (ER) known as 94-kDa glucose-regulated protein (Grp94). It is so called because it is induced by glucose starvation. Like Bip, Grp94 is also associated with unassembled immunoglobulin chains (Melnick *et al.*, 1992). However, immunoglobulin chains interact sequentially with these two ER stress proteins. They associate with Grp94 after Bip, until they are oxidized (Melnick *et al.*, 1994).

Being a molecular chaperone, HSP90 prevents aggregation by refolding of denatured proteins and plays a role in folding of newly synthesized proteins (Wiech *et al.*, 1992). HSP90 is involved in signal transduction pathways by interacting with cellular signalling proteins. These include several protein kinases such as viral tyrosine kinase $pp60^{src}$, "ligand-dependent" transcription factors like steroid hormone receptors and actin filaments.

After its synthesis in vertebrate cells, $pp60^{src}$, a protein-tyrosine kinase associates with two proteins, p50 and HSP90 which keep the kinase inactive. This

kinase is the product of viral oncogene v-*src* in Rous sarcoma virus and can transform cells. Once $pp60^{src}$ is inserted into the plasma membrane after myristyration at the amino(NH_2)-terminus, it is not associated with the HSP90 and p50 and becomes active as a kinase (Xu and Lindquist, 1993).

HSP90 regulates interactions between steroid hormones like cortisol and their receptors such as glucocorticoid receptor. HSP90 dimer binds to the steroid hormone receptor to form an aporeceptor complex and maintain the receptor in a transcriptionally inactive state in the absence of steroid hormone. Aporeceptor complexes are present in both cytoplasm and nucleus, depending on the type of steroid hormone receptor. Aporeceptor complexes of glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and dioxin receptor (DR) are in the cytoplasm, whereas aporeceptor complexes of androgen receptor (AR), estrogen receptor (ER) and progesterone receptor (PR) are in the nucleus (Bohen and Yamamoto, 1994). The intermediate complex consists of the steroid hormone receptor, an Hsp90 dimer and four partner proteins including HSP70, HSP40, HSP70 interacting protein (Hip) and HSP90-HSP70 organizing protein (Hop) (Fig. 1.1.). Hip (p48) is an HSP70 cofactor and Hop (p60 or Stil) is an assembly factor for HSP90 and HSP70 (Scheibel and Buchner, 1998). The intermediate complex soon dissociates and a mature complex is formed. In addition to the steroid hormone receptor and the HSP90 dimer, the mature complex contains a small highly acidic phosphoprotein called p23 and one of the three large immunophilins (IP/Hsp56) (FKBP51/p54/FKBP54, FKBP52/p56/p59/Hsp56/HBI and Cyp40). Immunophilins are ubiquitously expressed and highly conserved proteins that have peptidylprolyl isomerase (PPIase) activity. The binding of steroid hormone to the mature complex causes dissociation

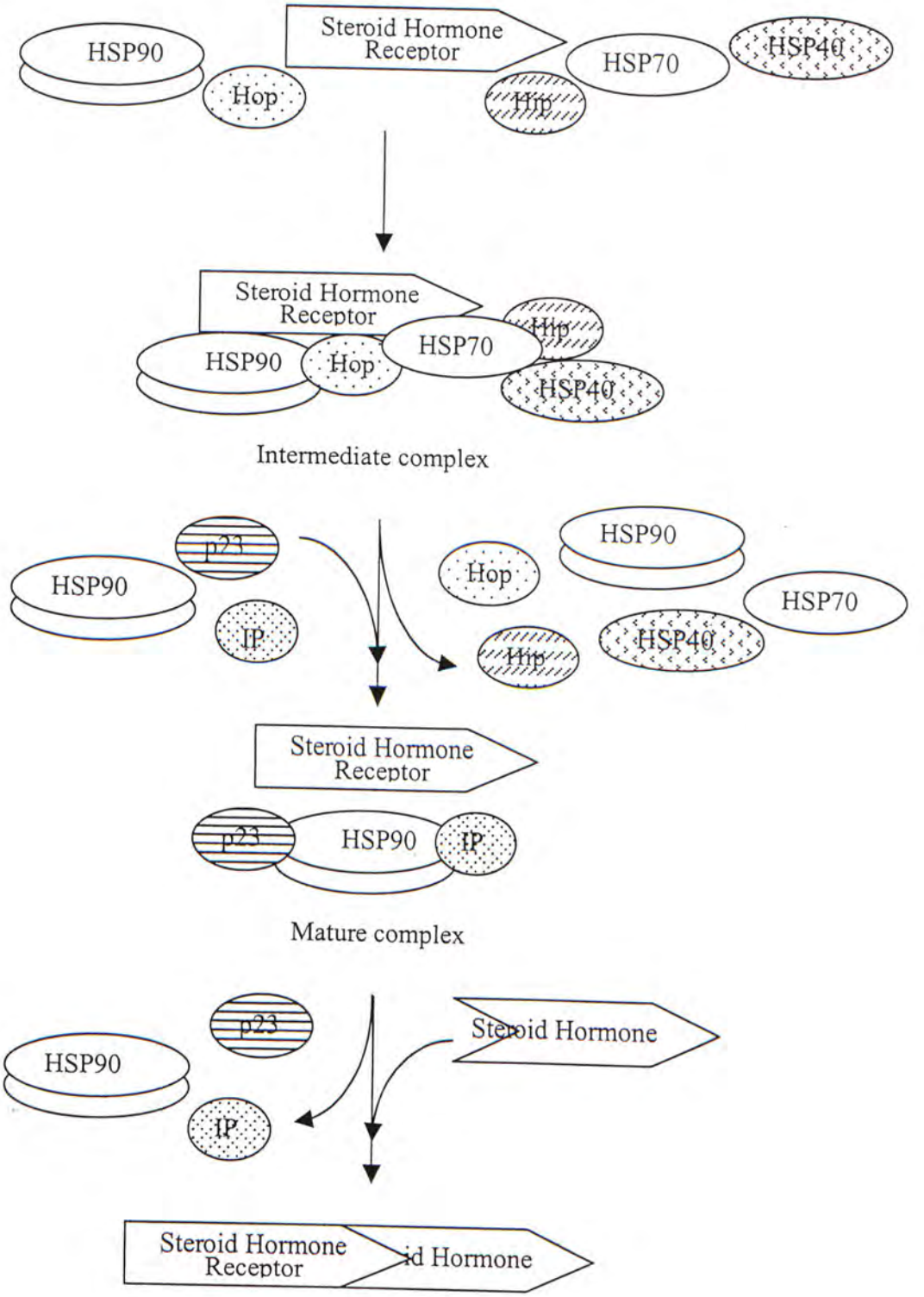

Figure 1.1. Binding of steroid hormone to the steroid hormone receptor of the mature complex (modified from Scheibel and Buchner, 1998).

of the complex. The steroid hormone receptor is now not associated with the HSP90 dimer any more. It dimerizes and binds to DNA where it alters the transcriptional activity.

HSP90 protein also binds to cytoskeletal subunit proteins such as actin and tunulin. However, Ca^{2+}-dependent binding of calmodulin to HSP90 inhibits the binding and cross-linking of HSP90 to actin filaments (Nishida *et al.*, 1986).

1.1.2.3. HSP70s

The 70-kDa heat shock proteins (HSP70s) are the most widely studied HSPs. They are present in all organisms. The bacterial homologue is DnaK and HSP70s are present in all cellular compartments of eukaryotes.

Four major members of the HSP70 family have been identified. The first member is hsp70 (also referred to as Hsp72) which is a cytosolic-nuclear protein induced by exposure to heat shock and other stresses. Another member is 70-kDa heat shock cognate (Hsc70 and also referred to as Hsp73). It is constitutively expressed in both cytosol and nucleus of normal unstressed cells and involved in "housekeeping" functions. Hsc70 is also called clathrin-uncoating ATPase. It disassembles the clathrin cages and releases clathrin from coated vesicles in an ATP-dependent manner. The third member is 78-kDa glucose regulated protein (Grp78) or immunoglobulin (Ig) heavy chain binding protein (Bip). It is found within the lumen of rough endoplasmic reticulum (ER). Lastly, within the matrix of mitochondria and chloroplasts 75-kDa

glucose regulated protein (Grp75, also referred to as mito-bip or mt-hsp70) is found. The last two members are both stress inducible and constitutively expressed (Welch *et al.*, 1998).

Proteins of HSP70 family is involved in many cellular processes, including folding of nascent proteins and refolding of denatured proteins, protein translocation across membranes of organelles, selective lysosomal proteolysis and regulation of heat shock response.

1.1.2.3.1. ATPase reaction cycle of HSP70 and protein folding

All HSP70 proteins contain an ATP-binding domain at the amino(NH_2)-terminus. This domain is more highly conserved in amino acid sequence than the peptide substrate-binding domain at the carboxy(COOH)-terminus. They all are peptide-unfolded protein-stimulated ATPases. The interaction of HSP70 with unfolded or denatured proteins is carried out via an ATP-dependent binding and release reaction cycle. Binding of ADP to the ATP-binding domain stabilizes binding of substrate protein or peptide at the carboxyl-terminus. This is followed by ADP/ATP exchange in the ATP-binding domain. K^+-dependent binding of ATP to the ATP-binding domain at the amino-terminus induces conformational changes in both the ATP-binding domain and the peptide substrate-binding domain. This results in the release of the substrate protein (Palleros *et al.*, 1993). The next step is ATP hydrolysis, i.e., release of P_i by cleavage of the -phsophate bond. The bound ADP induces another conformational change in the peptide substrate-binding domain.

There is a highly conserved protein folding machinery in the cytosol of both prokaryotes and eukaryotes and in eukaryotic mitochondria and chloroplasts to mediate the folding of oligomeric proteins to their native functional states. This protein folding machinery is composed of HSP70, and two cofactors, DnaJ (Hsp40) and GrpE. A nascent polypeptide on a ribosome at an early stage of translation interacts first with HSP70 and DnaJ to maintain its folding competence. Then, the newly synthesized protein is transferred by GrpE, a polypeptide release factor, to HSP60 and HSP10 for final folding. DnaJ and GrpE increase the ATPase activity of HSP70, with DnaJ accelerating the hydrolysis of the HSP70-bound ATP and GrpE accelerating the ADP/ATP exchange by increasing the release of HSP70-bound ADP (Liberek *et al.*, 1991).

1.1.2.3.2. Protein translocation

HSP70s facilitate protein translocation across membranes of organelles such as mitochondria and chloroplasts or through pores in the nuclear envelope. An unfolded nascent polypeptide emerging from a ribosome is bound to cytosolic HSP70 proteins (ct-hsp70) in the cytoplasm. This interaction prevents the growing polypeptide chain from folding incorrectly and prematurely. Also, HSP70 binds exposed hydrophobic sites of the unfolded protein to prevent aggregation with other proteins. To maintain the nascent or newly synthesized polypeptide in an unfolded or "translocation-competent" conformation across the outer and inner mitochondrial membranes cytosolic ATP/HSP70 or mitochondrial HSP70 (mt-hsp70) are required

(Wachter *et al.*, 1994). Both electrical membrane potential across the inner membrane and the binding of mt-hsp70 to the presequence of an incoming precursor in the matrix are required for trapping and stabilizing the presequence on the trans-side of the inner membrane (Ungermann *et al.*, 1994). For translocation across mitochondrial membranes, matrix ATP-dependent binding of mt-hsp70 to the precursor protein unfolds it outside the mitochondria by shifting the equilibrium of folded domains. The repeated Mim44-mediated reaction cycles of binding and release of mt-hsp70 from the incoming precursor chain drive the completion of translocation into mitochondria (Ungermann *et al.*, 1994). ATP hydrolysis destabilizes the interaction between Mim44 and mt-hsp70/precursor protein complex to allow the chain to diffuse through the channel. The mt-hsp70 then releases from the chain until a second mt-hsp70 binds to Mim44 and a next segment of the chain.

Within the lumen of endoplasmic reticulum (ER), immunoglobulin (Ig) heavy chain binding protein (BiP) that is associated non-covalently with unassembled Ig heavy chains is found (Haas and Wabl, 1983). This 78 kDa protein is also called glucose-regulated protein 78 (Grp78) as it is induced by glucose starvation and other stresses (Lee, 1992). In yeast, the Sec63-Bip complex plays an important role in post-translational protein translocation across the ER membrane (Brodsky et al., 1995). Sec63, a DnaJ homolog, is an integral membrane protein to target Bip to protein translocated into the lumen of the ER. Being a molecular chaperone, Bip is involved in ATP-dependent protein folding and assembly in the ER lumen. BiP interacts transiently with newly synthesized wild-type secretory proteins until folding and assembly are completed and more permanently with unassembled, misfolded, mutant and underglycosylated proteins (Gething and Sambrook, 1992).

1.1.2.3.3. Selective lysosomal proteolysis

The 73-kDa peptide recognition protein (prp73), identified as an Hsc70 (Chiang *et al.*, 1989), is involved in selective lysosomal proteolysis. Prp73 targets intracellular proteins to lysosomes for degradation and all these proteins contain specific peptide sequences biochemically related to Lys-Phe-Glu-Arg-Gln (KFERQ). Prp73 facilitates ATP-dependent selective uptake and degradation of the KFERQ motif-containing proteins by lysosomes through binding of the substrate proteins to lysosomal membrane proteins and then their import into the lysosomal lumen (Dice *et al.*, 1994).

1.1.2.4. HSP60s

HSP60 is also known as chaperonin 60 (cpn60). It is present only within eubacteria and organelles of endosymbiotic origin such as mitochondria and chloroplasts. HSP60 is highly conserved from prokaryotes to man and the bacterial homologue in prokaryotes is GroEL (Kabakov and Gabai, 1997).

HSP60 molecule is an oligomeric structure formed from 14 identical 60-kDa subunits and is arranged into two stacked heptameric rings, each enclosing a large central cavity ("double doughnut"). Each subunit contains an ATPase site (Soltys and Gupta, 1999).

HSP60 cooperates with a small protein, HSP10 (also referred to as chaperonin 10 or cpn10). It is a single heptameric ring of seven 10-kDa subunits. HSP10 is also

highly conserved and the bacterial homologue in prokaryotes is GroES. HSP10 forms a binary complex to bind asymmetrically to one end of the HSP60 ring in the presence of ATP (Viitanen *et al.*, 1992).

HSP60 or GroEL and HSP10 or GroES are required for Mg/ATP-, K^+-dependent folding by preventing aggregation and assembly of most proteins imported into mitochondria and synthesized within mitochondria. Exposed hydrophobic surfaces on the substrate protein bind to hydrophobic binding sites on one GroEL ring and this ring is ADP-bound. The tightly bound seven ADP molecules and hence the GroES cap are released from the GroEL ring. Seven ATP molecules then bind to the GroEL ring. ADP/ATP exchange causes binding of GroES cap to one end of GroEL ring. However, the ATP-bound form has the lowest substrate affinity (Bochkareva *et al.*, 1992). This results in release of the bound substrate protein into the central cavity of the GroEL ring for folding. GroES increases the cooperativity of ATP binding and hydrolysis (Bochkareva *et al.*, 1992). Cooperative hydrolysis of seven ATPase molecules by binding them to the seven subunits within one GroEL ring also causes release of the GroES cap from the ring. The substrate protein is therefore allowed to exit. If folding has not been completed and exposed hydrophobic surfaces can still be found on the substrate protein, it will rebind to a GroEL ring and another cycle of binding and release begins (Soltys and Gupta, 1999).

1.1.2.5. Small HSPs

Heat shock proteins are subdivided into two groups based on their molecular

weight: the large or high-molecular-weight heat shock proteins and small or low-molecular-weight (LMW) heat shock proteins (sHSPs). Large heat shock proteins include all members of HSP100, HSP90, HSP70 and HSP60. sHSPs are heat shock proteins of molecular weight from 16 to 30 kDa. They are found in all organisms, but their number in different species is variable. For example, there are only two sHSPs in *Escherichia coli* (IpbA and IpbB) (Allen *et al.*, 1992). Three sHSPs were identified in yeasts. Mammals have three sHSPs including homologous αA-crystallin and αB-crystallin from eye lens. Fruit flies *Drosophila* have four sHsps. There are more than 20 sHSPs in plants (Arrigo and Landry, 1994) and they can be found in cytosol and different organelles such as chloroplasts, mitochondria and endoplasmic reticulum (ER) (Ehrnsperger *et al.*, 1998). sHSPs are less conserved than large heat shock proteins but all members of this family contain a conserved α-crystallin domain in the carboxy(COOH-) terminal half.

Two main characteristics of sHSPs are oligomerization and phosphorylation. All sHSPs have the tendency to form large oligomeric structures with molecular mass up to 800kDa or more and oligomerization can be increased by increasing intracellular glutathione level (Mehlen *et al.*, 1997). Some sHSPs, like bovine A-crystallin and B-crystallin, rat Hsp25, human Hsp27 and mouse Hsp25, are phosphorylated by stimuli including heat shock, mitogens such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), inflammatory cytokines such as tumor necrosis factor (TNF-) and interleukin-1 (IL-1), and agents inducing oxidative stress such as arsenite and hydrogen peroxide. In all these sHSPs, serines are the exclusively phosphorylated amino acid residues (Ehrnsperger *et al.*, 1998). The serine residues of Hsp25 and Hsp27 are phosphorylated by mitogen-activate protein kinase-activated

protein (MAPKAP) kinase 2 and 3pk (or 3) (Ludwig *et al.*, 1996).

sHSPs also protect cytoskeletal architecture. Murine Hsp25 inhibits actin polymerization (Miron *et al.*, 1991) and α-crystallins modulate intermediate filament assembly (Nicholl and Quinlan, 1994). Phosphorylation of Hsp27 stabilizes actin microfilament organization by conferring cellular resistance to heat shock (Lavoie *et al.*, 1995). On the contrary, only unphosphorylated murine Hsp25 has actin polymerization-inhibiting activity in vitro (Benndorf *et al.*, 1994).

Unlike large heat shock proteins, sHSPs act as molecular chaperones in an ATP-independent manner. α-crystallins prevent non-specific aggregation of proteins denatured by heat in mammalian eye lens (Rao *et al.*,1995). sHSPs like Hsp25, Hsp27 and α-crystallin can also induce renaturation of denatured proteins (Jakob *et al.*, 1993).

1.1.2.6. Ubiquitin

Ubiquitin (Ub) is a highly conserved protein composed of 76 amino acids occurred in all eukaryotes but not in prokaryotes. *UB14*, a yeast poly-Ub gene that encodes five head-to-tail ubiquitin repeats, can be induced by heat shock (Finley *et al.*, 1987) and other stresses, ubiquitin is therefore also regarded as a heat shock protein. Ub is the smallest heat shock protein with molecular weight of about 8 kDa and is involved in the ubiquitin-dependent proteosome pathway for selective protein degradation.

The ubiquitin-dependent proteasome pathway is the major pathway in eukaryotic cells for the degradation of abnormal proteins damaged by stress. Proteasome is a 26S ATP-dependent protease complex. The 26S proteasome consists of a 20S proteasome and a 19S regulatory complex (Coux *et al.*, 1996). The cylindrical 20S proteasome consists of a stack of heptameric rings. The outer ring at each end of the cylinder is composed of seven α-type subunits and each of the two inner rings is formed from seven β-type subunits. Six ATPase subunits are identified in the 19S regulatory complex. To degrade substrate proteins by 20S proteasome, they should be unfolded before access through the narrow pore of the outer rings into the active sites in the inner rings of β-type subunits, and the unfolding of substrate proteins requires ATP (Pickart, 1999).

Ubiquitin is covalently conjugated to the substrate protein by three enzymes. First, ubiquitin-activating enzyme (E1) activates the carboxy(COOH)-terminus of ubiquitin to form an E1-ubiquitin thiol ester in an ATP-dependent reaction. Activated ubiquitin is then transferred to an active cysteine residue of a ubiquitin-conjugating enzyme (E2). Finally, ubiquitin is transferred from the E2 and attached to the substrate protein through an isopeptide linkage between the glycine residue at the carboxy(COOH)-terminus of ubiquitin and the lysine residue of the substrate protein, with the aid of ubiquitin-protein ligase or recognin (E3). For the substrate protein to be recognized and degraded by the 26S proteasome, a signal in the form of a poly- or multi-ubiquitin chain in which multiple ubiquitin molecules are linked together with isopeptide linkages, by E2, is required (Pickart, 1997). After the substrate protein has been degraded by the 26S proteasome, the ubquitin molecules are released.

1.1.3. HSP studies in fish

Studies of HSP expression in fish are divided into *in vivo* and *in vitro* works. *In vivo* works involve the intact fish and *in vitro* works deal with fish cell lines, primary cultures of fish cells and tissues extracted from the fish (Iwama *et al.*, 1998).

1.1.3.1. *In vivo* works

In vivo works involve the intact fish and most of them have studied the effects of heat shock (Iwama *et al.*, 1998). Hepatic HSP70 levels of *Sparus sarba* were shown to elevate 1 hour after exposing the fish to a temperature of 7° C higher than the ambient (Deane *et al.*, 1999a, 2000a). Heat shock at 25° C caused significant increases in amounts of HSP70 mRNA in blood, brain, heart, liver, red and white muscles of rainbow trout significantly (*Oncorhynchus mykiss*) (Currie *et al.*, 2000). In this study, amounts of HSP30 mRNA in these tissues were also investigated. Except the blood, HSP30 mRNA abundance in all tissues increased following the *in vivo* heat shock (Currie *et al.*, 2000). In another study, four species of marine teleost fishes, buffalo sculpin (*Enophrys bison*), speckled sanddab (*Citharichthys stigmaeus*), English sole (*Parophrys vetulus*) and Pacific staghorn sculpin (*Leptocottus armatus*), were subjected to a heat-stress regime. This resulted in induction of both HSP70 and HSP90 synthesis in their tissues (Dietz and Somero, 1993).

Fish exposed to environmental contaminants have also been shown to increase expression of heat shock proteins in their tissues. In a study on rainbow trout, intraperitoneal injection of β-naphthoflavone (BNF) induced hepatic HSP70

expression (Vijayan *et al.*, 1997a). Induction of hepatic HSP70 expression was also observed in rainbow trout following the exposure of the fish to sublethal concentrations of acutely toxic bleached kraft pulp mill effluent (BKME) or sodium dodecylsulphate (SDS) for 96 hours (Vijayan *et al.*, 1998). In the same study, exposing the swimming chinook salmon (*Oncorhynchus tshawytscha*) to sublethal concentrations of treated BKME for 30 days has also been shown to increase the hepatic HSP70 expression (Vijayan *et al.*, 1998).

Pathogen invasions has also reported to have induced the expression of heat shock proteins. In a study on coho salmon (*Oncorhynchus kisutch*), fish was induced experimentally by injection with *Renibacterium salmoninarum*. Fish infected with bacterial kidney disease (BKD) had higher levels of HSP70 in both kidney and liver than the control fish (Forsyth *et al.*, 1997). In sea bream *Sparus sarba* infected either naturally or experimentally by *Vibrio alginolyticus*, augmented HSP70 expression has been noted in many tissues, including the macrophages, liver and gill (Woo *et al.*, 1999).

The effects of long-term (8 months) salinity adaptation on black sea bream (*Mylio macrocephalus*) on expression of various heat shock proteins were examined. Hepatic HSP90, HSP70 and HSP60 levels were shown to be lowest in fish reared in an iso-osmotic salinity (12 ppt) and highest at salinity extremes of 6 ppt and 50 ppt (Deane *et al.*, 2001a).

The effects of some hormones on HSP expression in fish have also been addressed. In a study on *Sparus sarba*, daily intraperitoneal injections of

recombinant bream growth hormone (rbGH) and ovine prolactin (oPRL) were shown to reduce hepatic HSP70 levels significantly (Deane *et al.*, 1999a). The downregulation of HSP70 expression suggested that growth hormone and prolactin might be related to a reduced stress state. In addition, the effect of two pharmacological drugs that can regulate the endogenous prolactin synthesis on hepatic HSP70 expression in silver sea bream was investigated (Deane *et al.*, 2000a). Daily intraperitoneal injections of sulpiride, a prolactin stimulant, caused a decrease in hepatic HSP70 levels. In contrast, administration of bromocriptine, a prolactin suppressant, increased the levels of hepatic HSP70 significantly. The results reinforced the notion that prolactin might have a protective effect on stress tolerance in fish. However, daily administration of exogenous cortisol was found to have no effect on hepatic HSP70 expression (Deane *et al.*, 1999a). Although cortisol is a classic stress hormone and HSP70 is a cellular stress response protein, cortisol appeared to be unrelated to the piscine HSP70 expression.

1.1.3.2. *In vitro* works

In vitro works involve fish cell lines, primary cultures of fish cells and tissues extracted from the fish. Like *in vivo* works, most of the *in vitro* works investigated the effect of hyperthermia. For example, *in vitro* heat shock at 25°C resulted in significant increases in mRNA of both HSP70 and HSP30 in whole blood of the rainbow trout (Currie *et al.*, 2000). The HSP70 synthesis in rainbow trout red blood cells can be blocked by the transcriptional inhibitor actinomycin D, suggesting the synthesis of HSP70 was regulated at the level of transcription (Currie *et al.*, 1997).

The levels of 67, 69 and 92 kDa in primary cultures of hepatocytes and gill epththelial cells of rainbow trout, and a fish cell line called fibroblast-like RTG-2 cells were found to elevate after being exposed at 26°C (Airaksinen *et al.*, 1998). However, the levels of Hsp104 were found to increase in RTG-2 cells only. Cold shock was also demonstrated to induce HSP70 expression in fish. In the study of Yamashita *et al.* (1996), cold shock at 4°C induced the synthesis of a 70kDa protein in rainbow trout cell line RTG-2. It was thought that the induction was a metabolic compensation for the delay in cell cycling due to low temperatures. The authors also suggested that the 70kDa protein might be directly involved in cold acclimation.

In vitro exposure to other stresses such as environmental contaminants, hypoxia and osmotic stress also induced the synthesis of heat shock proteins. Cultured epidermal cells from explants of skin of rainbow trout showed an increase in the expression of heat shock proteins after exposure to sublethal concentrations of cadmium (Lyon *et al.*, 1998). Low oxygen tension of 1% O_2 increased the synthesis of 36, 39 and 51 kDa heat shock proteins in primary cultures of rainbow trout gill epithelial cells (Airaksinen *et al.*, 1998).

Osmotic stress was also shown to induce the expression of heat shock proteins in tissues extracted from the fish. In one study, HSP70 was induced in the isolated branchial lamellae of anadromous Atlantic salmon (*Salmo salar*) was exposed to *in vitro* hyper-osmotic conditions of either NaCl or glycerol (Smith *et al.*, 1999). In the study by Pang *et al.* (2000), quantity of HSP90 mRNA in isolated salmon branchial lamellae increased after *in vitro* exposure of the tissue to hyper-osmotic conditions. These *in vitro* hyper-osmotic conditions had no effect on the HSP90 expression in

kidney tissue. In spite of this, *in vitro* thermal stress could cause an increase in the quantity of HSP90 mRNA in both branchial lamellae and kidney tissue. The authors suggested that transcription of HSP90 mRNA in response to osmotic stress might be unrelated to denaturation of cellular proteins and that HSP90 protein synthesis may be regulated at both the transcription and translation levels (Pan *et al.*, 2000).

The effect of cortisol on the HSP90 mRNA expression in primary culture of rainbow trout hepatocytes was also examined. In this study, exposure of trout hepatocytes to cortisol for 24 hours at ambient temperature failed to affect HSP90 mRNA expression. However, high level of cortisol (1000ng/ml) was found to attenuate the HSP90 mRNA expression induced by heat shock. The authors suggested cortisol regulated transcription of heat shock proteins only when the levels of the classic stress hormone were high, i.e., under stressful conditions (Sathiyaa *et al.*, 2001).

1.2. Growth hormone / prolactin family in teleostean fishes

1.2.1. Introduction

Hormones are specific chemical substances that are produced by the brain and the endocrine glands and then secreted into the blood through which they are transported to a distant target organ, tissue or group of cells. Within the target organ, tissue or group of cells, the hormone binds to a specific receptor molecule. The binding of hormone to receptor stimulates the release of an intracellular second messenger molecule that amplifies the hormonal signal (Norris, 1997b).

The growth hormone / prolactin family includes growth hormone (GH; somatotropin), prolactin (PRL), somatolactin (SL) and placental lactogen (PL; somatomammotropin). Growth hormone and prolactin are found in all vertebrates. Somatolactin is confined to teleosts while placental lactogen is a mammalian hormone in the placenta. Growth hormone, prolactin and somatolactin are produced in the adenohypophysis of pituitary. All members are protein hormones of similar structure and amino acid sequence. All of these hormones are single globular polypeptide chains that are composed of about 200 amino acids and folded by intramolecular disulfide bridges. They have a small loop at the carboxy(COOH)-terminus and a big loop formed by three-quarters of the remaining amino acids (Nicoll *et al.*, 1986). They are large proteins with molecular mass of about 20 to 28 kDa. These similarities contribute to the common biological actions shared by these hormones. The hormones of this family are evolved from a common ancestral protein.

Growth hormone transcription and secretion are primarily controlled by two hypothalamic factors. They are stimulated by growth hormone-releasing hormone (GHRH) or factor (GRF) and inhibited by somatostatin (somatotropin release-inhibiting factor / SRIF or hormone / SRIH). GRF receptor and somatostatin receptor are all specific G protein-coupled membrane receptors. There are five subtypes of somatostatin receptor (sst1-5). Subtypes sst1, 2 and 5 inhibit growth hormone secretion while sst2 and sst5 inhibit glucagon secretion and insulin secretion respectively (Benali *et al.*, 2000).

The major control of prolactin release is inhibitory from the hypothalamus. Prolactin release is inhibited by dopamine (DA), a catecholamine. However, dopamine can stimulate the release of growth hormone in goldfish (Wong *et al.*, 1992). Thyroid-stimulating hormone (TRH) from hypothalamus can stimulate prolactin release but it can also stimulate thyroid-stimulating hormone (TSH; thyrotropin) release.

The adenohypophysis of teleosts is divided into three main zones. They are the anterior pars distalis and posterior pars intermedia. The pars distalis of teleosts is further divided into the rostral and the proximal zones. Each adenohypophysial tropic hormone is produced by a distinct endocrine cell type in a particular region of adenohypophysis. The cells are mainly divided into two types: acidophils and basophils. Acidophils are cells with granules that can be stained by acidic dyes and basophils are cells with granules that can be stained by basic dyes. The cells that produce growth hormone and prolactin are acidophils. The acidophils that secrete growth hormone are called somatotropes while the acidophils that secrete prolactin are

known as lactotropes (Norris, 1997b).

There are seven adenohypophysial tropic hormones in teleosts and they fall into three chemical categories based on their amino acid sequences. Category 1 is pituitary glycoprotein hormones including two gonadotropin hormones (GTH-I and GTH-II) and thyroid-stimulating hormone. These hormones consist of two peptide subunits, and subunits, and are produced in proximal pars distalis. Category 2 is the growth hormone / prolactin family. Growth hormone, prolactin and somatolactin belong to this category. Somatotrope and lactotrope cells are located in proximal pars distalis and rostral pars distalis respectively. Somatolactin is produced from PAS(+) or PIPAS cells. The type of cells is so called because they are present in pars intermedia that can be stained with periodic acid-Schiff (PAS) reagent. Category 3 is the proopiomelanocortin (POMC)-gene derived peptides. These include corticotropin or adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormone (MSH). Both these hormones are derived from a common precursor molecule or prohormone known as POMC. ACTH cells are found in rostral pars distalis and MSH cells are present in pars intermedia (Norris, 1997b).

1.2.2. Growth hormone (GH; somatotropin)

1.2.2.1. Structure

Growth hormone is a protein hormone produced by the proximal pars distalis of adenohypophysis. It is stored in the somatotrope cells as a dimer complexed with zinc and released as a monomer in the blood (Carter-Su *et al.*, 1996). Similar to the big loop, little loop pattern of human growth hormone molecule, there are two disulfide bridges in a fish growth hormone molecule, such as the one identified in chum salmon (Vestling *et al.*, 1991). Two structural variants of growth hormone have been identified in teleosts. For instance, both variants of Atlantic cod growth hormone have 185 amino acids but they differ from each other by only one amino acid in terms of their sequences (Rand-Weaver *et al.*, 1991).

1.2.2.2. Actions

Growth hormone is famous for its growth-promoting effects. These effects can be shown in salmon raised in aquaculture. Either implantation of somatotropin-containing slow-release pellets or oral administration of bovine somatotropin accelerated the growth in coho salmon (McLean *et al.*, 1990, 1992).

Growth hormone is protein anabolic because it stimulates uptake of amino acids and protein synthesis and inhibits nitrogen excretion, especially in skeletal muscle and bone. Growth hormone also promotes fat mobilization by increasing lipolysis and

free fatty acid oxidation and decreasing lipogenesis. Growth hormone is also important in carbohydrate metabolism, but this is related to its actions on lipid and protein metabolism. Due to fatty acid oxidation, glycolysis and glycogenesis are inhibited. This leads to increased blood glucose concentrations (hyperglycemia) and hence insulin secretion and deposition of glycogen (Bentley, 1998a).

Growth hormone is also involved in regulating piscine osmoregulation. This has been intensively studied in salmonids, but recent data also indicate a similar role for other teleostean species. Increased plasma growth hormone concentrations are important in adaptation to seawater during parr-smolt transformation in salmons (Young *et al.*, 1989). The hormone also stimulates proliferation of gill chloride cells and increases Na^+–K^+–ATPase ("sodium pump") activity that is the driving force for monovalent ion transport in chloride cells (Prunet *et al.*, 1989). The chloride cell density in the opercular epithelium of tilapia almost doubled after treatment with growth hormone (Flik *et al.*, 1993). Growth hormone acts in synergism with cortisol to increase gill Na^+–K^+–ATPase activity (McCormick, 1996). Shrimpton *et al.* (1995) found that growth hormone treatment increased the number of gill glucocorticoid receptors in juvenile coho salmon. Growth hormone even increases cortisol secretion by stimulating the interrenal gland or enhancing the sensitivity of the interrenal to ACTH (Hazon and Balment, 1998). In fact, growth hormone has a permissive effect on ACTH in adrenocortical secretion in rats (Sonntag, 1996). The roles of growth hormone in metabolism and osmoregulation are very important in sexual maturation during parr-smolt transformation in salmonids (Bjornsson, 1997).

Growth hormone exerts its effects on target tissues directly through specific

receptors on the plasma membrane or indirectly through its peptide mediators, insulin-like growth factors (IGFs) or somatomedins (SMs). For instance, growth hormone was found to increase the plasma levels of IGF-I in gilthead seabream (Funkenstein *et al.*, 1989). In growing coho salmon, increased hepatic IGF-I mRNA levels followed the increase in plasma growth hormone (Duan *et al.*, 1995). As with growth hormone, IGF-I also improves growth. The 2-year-old juvenile coho salmon received implants of osmotic minipumps containing recombinant bovine insulin-like growth factor-I (rbIGF-I) for 25 days was reported to exhibit a doubled linear growth rate and an increased growth in weight by 40% (McCormick *et al.*, 1992). IGF-I stimulated the synthesis of muscle protein of gulf killifish as evidenced by enhanced incorporation of [^{14}C]glycine (Negatu and Meier, 1995). Also, injection of IGF-I has the same effect as growth hormone in stimulating salinity tolerance in rainbow trout (McCormick *et al.*, 1991).

1.2.2.3. Insulin-like Growth Factors (IGFs; Somatomedins)

There are two insulin-like growth factors (IGFs): IGF-I and IGF-II. IGF-I is the form of IGF produced in adults and IGF-II is a fetal growth factor (Bentley, 1998b). IGF-I was called "sulfation factor" or "somatomedin C" because IGF-I was found to cause incorporation of sulfate into cartilage leading to growth. IGF-I is a polypeptide composed of 70 amino acids. It is mainly synthesized in the liver and also other tissues. The most important secretogogue is growth hormone and plasma IGF-I regulates growth hormone release through negative feedback mechanism at the levels of hypothalamus and pituitary. IGF-I stimulates somatostatin release and inhibits

growth hormone-releasing hormone release. In fact, these four hormones form the growth hormone-releasing hormone-somatostatin-growth hormone-IGF-I axis (Fig. 1.2.).

IGFs belong to the insulin family of peptides. They are structurally related to insulin. In fact, IGFs have both growth-promoting effects and insulin-like activity. IGF-I is highly conserved in amino acid sequence. IGF-I of human, cattle and pigs are identical. IGF-I of sheep differs from that of human IGF-I by only 1 amino acid substitution. IGF-I of rat and mouse differ from that of human by 3 and 4 amino acid substitutions respectively. IGF-I of human differs from those of domestic fowl, toad and Atlantic fish by 8, 11 and 14 amino acid residues respectively (Bentley, 1998b).

Both IGF-I and IGF-II bind to specific high-affinity carrier proteins, insulin-like growth factor binding proteins (IGFBPs) in blood plasma to form a complex. IGFBPs can be found in all vertebrates including teleosts. IGFBPs prolong the half-life of IGFs and control the activities of IGFs by preventing the receptor binding. IGFBPs can be negative growth regulators. When zebrafish insulin-like growth factor binding protein 2 (IGFBP2) was added to cultured zebrafish and mammalian cells, cell proliferation and DNA synthesis stimulated by IGF-I were significantly inhibited (Duan *et al.*, 1999).

Receptors for IGF-I and insulin belong to the same family. Each IGF-I receptor is a heterotetramer that consists of two α-subunits and two β-subunits linked by disulfide bridges and has intrinsic tyrosine kinase activity of which the IGF-II receptor lacks. The α-subunit is the extracellular hormone (ligand)-binding recognition site.

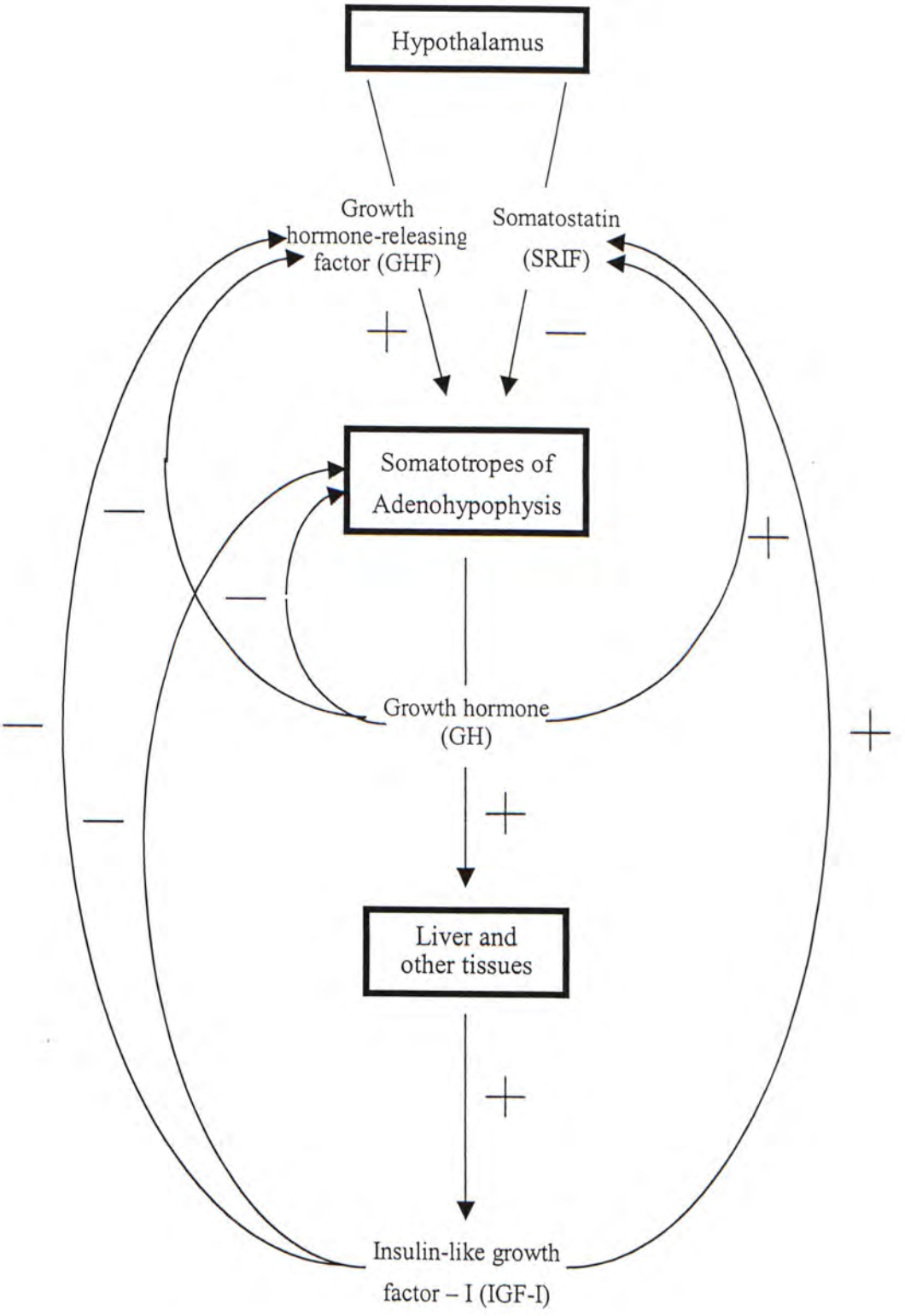

Figure 1.2. The growth hormone-releasing hormone-somatostatin-growth hormone-IGF-I axis (modified from Batten and Ingleton, 1987).

The β-subunit contains extracellular, transmembrane and the cytoplasmic or intracellular tyrosine kinase domains. Binding of IGF-I to the extracellular recognition site results in autophosphorylation of the intracellular tyrosine kinase domain. The tyrosine kinase is now activated and is able to phosphorylate tyrosine residues of insulin receptor substrate (IRS) family of proteins which mediate intracellular signaling by IGF-I and insulin (Mommsen, 1998).

1.2.3. Prolactin (PRL)

1.2.3.1. Structure

Prolactin is a protein hormone produced by the lactotrope cells of the rostral pars distalis of adenohypophysis. Prolactin is a single polypeptide chain with a molecular mass of about 23 kDa and of about 200 amino acids. However, there are fewer amino acids in prolactin molecules of some fish. Prolactins in teleosts have only two disulfide bridges while there are three in most vertebrates including dipnoans (lungfishes) (Bentley, 1998b). Some species have two forms of prolactin, for example, the teleost tilapia. Tilapia prolactins have sequence identity of only 69% of each other. One has 188 amino acids (tilapia prolactin-188; PRL-I) and the other has 177 amino acids (tilapia prolactin-177; PRL-II). However, they are produced in the same cells and stored in the same granules (Specker *et al.*, 1993).

1.2.3.2. Actions

Prolactin is a versatile protein hormone and its functions are different among diverse groups of vertebrates. Its functions are related to five broad categories: reproduction, growth and development, osmoregulation, maintenance of integumentary structures and actions on steroid-dependent target tissues or synergisms with steroid hormones (Norris, 1997a).

In mammals, prolactin stimulates growth and development of mammary glands and increases milk secretion or lactogenesis by synthesis of milk fatty acids, milk proteins (casein and lactalbumin) and milk sugar, lactose. During lactation, the stimulus of prolactin secretion is suckling on the nipple. Prolactin also induces a specialized exocrine gland called crop sac of pigeons and doves to secrete pigeon- or crop-milk with which they feed their young by proliferation of the crop-sac epithelium. Prolactin also stimulates the skin of a discus fish, a cichlid, to secrete mucus called discus "milk" for the young to feed on. The maintenance of ventral brood pouch called marsupium for incubating developing eggs in male seahorses requires prolactin. The marsupial epithelium proliferates and secretes a protease to digest the yolk of the embryos (Ball, 1969).

Prolactin also plays a role in regulation of gonadal steroidogenesis in teleosts. Purified chum salmon prolactin increased plasma levels of testosterone in male hypophysectomized *Fundulus*. This demonstrates that prolactin has a gonatotropic action in teleosts. Also, it stimulated testosterone production by testes in male hypophysectomized *Fundulus*. This shows that prolactin has a steroidogenic action

in teleosts (Singh *et al.*, 1988).

Prolactin is very important in osmoregulation in freshwater teleosts. Injection of prolactin prolongs the survival of hypophysectomized euryhaline fish in fresh water by preventing excessive loss of Na^+ across the gills (Lam, 1972). Plasma prolactin levels decrease in euryhaline fishes after they are transferred from fresh water to seawater (Hazon and Balment, 1998). Prolactin was shown to decrease gill Na^+–K^+–ATPase activity but increase kidney Na^+–K^+–ATPase activity in hypophysectomized freshwater killifish (Pickford *et al.*, 1970). In a study on euryhaline teleost *Sparus sarba*, a significant reduction of branchial Na^+–K^+–ATPase alpha-subunit mRNA levels was observed in both seawater and hypo-osmotic conditions after prolactin treatment (Deane *et al.*, 1999b). Prolactin also reduces the permeability of the epithelia of osmoregulatory organs such as gills, skin, intestine and urinary bladder and kidneys to ion and water. Prolactin increases mucus secretion from skin, gills and buccal epithelium. The renotropic action of prolactin increases the number and the size of glomeruli of kidney epithelia (Norris, 1997a). Prolactin was also reported to affect branchial chloride cells. Prolactin was thought to reduce chloride cell numbers (Foskett and Scheffey, 1982). Chloride cell size and cell height were reduced in seawater-adapted tilapia following daily injections of prolactin (Herndon *et al.*, 1991). However, there is no significant change in chloride cell density.

Hypophysectomy causes hypocalcemia in teleosts like killifish *Fundulus* (Pang, 1973). Injection of ovine prolactin increased the branchial uptake of calcium ions in tilapia in fresh water (Flik *et al.*, 1986). The actions of prolactin are increasing gill

Ca-ATPase activity and stimulating proliferation of chloride cells (Flik *et al.*, 1993). Homologous recombinant tilapia prolactins I and II also have this hypercalcemia effect (Flik *et al.*, 1994). In fact, prolactin was released in *Gasterosteus* in fresh water with a low calcium concentration (Wendelaar Bonga, 1978). Since prolactin was shown to increase the plasma cortisol levels in a dose-dependent manner in tilapia, prolactin is thought to have a corticotropic effect on the interrenal tissue in tilapia (Flik *et al.*, 1994). In this way, prolactin and cortisol may act synergistically to control osmoregulation of teleosts in fresh water.

1.2.4. Somatolactin (SL)

1.2.4.1. Structure

Somatolactin is produced from PAS(+) cells, a cell type can be stained with periodic acid-Schiff (PAS) reagent. They are present in the pars intermedia of telsosts, so they are also called the PIPAS cells. Somatolactin molecules are composed of 204 to 209 amino acids and have three disulfide bridges. There are two forms of somatolactin: one is glycosylated and the other is non-glycosylated, such as somatolactin in Atlantic cod (Rand-Weaver *et al.*, 1991) and gilthead sea bream (Cavari *et al.*, 1995). Somatolactin is a highly conserved protein. The identities of somatolactin in terms of amino acid sequence among Atlantic cod, chum salmon and flounder were between 73 and 81%. For the corresponding growth hormones, the identities were only from 58 to 62% (Takayama *et al.*, 1991).

1.2.4.2. Actions

The physiological functions of somatolactin remain unclear, but hypercalcemic action may be one of the functions of somatolactin. The activity of somatolactin cells in terms of nuclear areas of somatolactin cells, somatolactin mRNA abundance and plasma somatolactin levels in the rainbow trout were found to increase when the rainbow trout were transferred to low calcium environment (Kakizawa *et al.*, 1993). Somatolactin may also be involved in acid-base regulation. Plasma somatolactin levels in rainbow trout were elevated during acidosis induced by exposure to acidic water or by exhaustive exercise (Kakizawa *et al.*, 1996). On the other hand, somatolactin stimulated *in vitro* gonadal steroidogenesis in coho salmon in terms of production of 11-ketotestosterone and testosterone by testicular fragments and production of estradiol by ovarian follicles (Planas *et al.*, 1992). Somatolactin may also play a role in mediating the stress response, especially in salmonids. Handling and confinement stress caused increases in plasma somatolactin levels in chinook salmon and rainbow trout (Rand-Weaver *et al.*, 1993). Acute stress and exhaustive exercise resulting from being chased in shallow water increased the plasma somatolactin concentrations in rainbow trout (Kakizawa *et al.*, 1995). Somatolactin may be involved in adaptation to dark backgrounds in sciaenid fishes. The plasma somatolactin levels in both red drum and Atlantic croaker exposed to dark backgrounds were significantly higher than in those exposed to light backgrounds (Zhu and Thomas, 1995). Also, plasma somatolactin concentrations were significantly increased in red drum after being transferred from light to black background tanks (Zhu and Thomas, 1996).

1.2.5. Growth hormone (GH-R) and prolactin receptors (PRL-R)

Growth hormone (GH-R) and prolactin receptors (PRL-R) belong to growth hormone / prolactin / cytokine receptor family. This family has two main characteristics. The first one is the two pairs of cytokines at the amino(NH_2)-terminus and the second one is the highly conserved WSXWS motif (tryptophan, serine, any amino acid, tryptophan, serine) at the carboxy(COOH)-terminus (Kelly *et al.*, 1995).

Like other receptors, GH-R and PRL-R have extracellular, transmembrane and cytoplasmic domains. Two molecules of human growth hormone receptors (hGH-R) form a homodimer as they bind to a molecule of growth hormone. There are two receptor sites, sites 1 and 2, on a hGH molecule and the two hGH-R molecules interact sequentially with the two sites on the hGH molecule (De Vos *et al.*, 1992). Homodimerization of the growth hormone receptor is a characteristic in both mammals and fish (Calduch-Giner *et al.*, 1997).

There is evidence that PRL-R also forms a dimer on binding, for instance, in intact Nb-2 rat lymphoma cell line, the prolactin receptor that binds to ovine placental lactogen exists as a dimer (Sakal *et al.*, 1997).

The JAK-2-STAT pathway is involved in the signal transduction of GH-R and PRL-R. JAK2 belongs to the family the Janus kinase (JAK) family of tyrosine kinase. All members of this family have 2 kinase domains. Receptor dimerization induces tyrosine phosphorylation of the receptor itself and the prebound JAK2, which, in turn,

phosphorylates signal transducers and activators of transcription (STAT) (Hooghe-Peters and Hooghe, 1995). Two highly related STATs are activated by cytokines: STAT5a for prolactin signaling and STAT5b for growth hormone signaling (Heim, 1999). Activated STAT forms either homodimer or heterodimer and is then translocated into the nucleus where it binds to specific DNA elements in promotors of target genes (Heim, 1999).

1.3. Cortisol in teleostean fishes

Cortisol is the major corticosteroid in the majority of teleost fish species. It is a 21-C steroid produced by the interrenal tissue. The interrenal tissue of teleosts is homologous to the adrenal cortex of mammals. Cortisol in teleosts plays the roles of both glucocorticoid and mineralocorticoid in mammals. Cortisol is a classic stress hormone, but it is also involved in intermediary metabolism and osmoregulation of teleosts (Wendelaar Bonga, 1997).

Stressors, either acute or chronic, increase the plasma cortisol levels by activation of the hypothalamo-pituitary-interrenal (HPI) axis. Under stressful conditions, a hypothalamic factor, corticotropin-releasing hormone (CRH), a neuropeptide, stimulates the release of a tropic hormone, corticotropin or adrenocorticotropic hormone (ACTH), from corticotropes in rostral pars distalis of adenohypophysis, which in turn, stimulates the secretion of cortisol from the interrenal tissue. Cortisol copes with stress by mobilization of energy reserves and maintenance of ionic balance (Hazon and Balment, 1998). Plasma cortisol is used as a primary stress response parameter. Elevation of plasma cortisol is a reliable and the most widely used indicator of stress in fish (Wendelaar Bonga, 1997). In the unstressed teleost tilapia, the plasma level of cortisol was about 20ng/ml. Two hours of confinement stress raised the plasma cortisol concentration to over 100ng/ml (Vijayan *et al.*, 1997b).

The glucocorticoid role of cortisol in fish was also shown in the studies on the eel (*Anguilla japonica*) (Chan and Woo, 1978) and coastal cutthroat trout

(*Oncorhynchus clarki clarki*) parr (Morgan and Iwama, 1996). In both these studies, administration of cortisol was found to increase the oxygen consumption and plasma glucose levels significantly. In intermediary metabolism, cortisol causes hyperglycaemia by promoting gluconeogenesis and glycogenesis in the liver. It promotes gluconeogenesis by increasing the activity of liver transaminase enzymes such as glutamate-pyruvate transaminase for deamination of amino acids while it promotes glycogenesis by increasing the activity of glycogen synthetase (Bentley, 1998, Hazon and Balment, 1998).

Cortisol is important in osmoregulation in both marine and freshwater teleosts. Cortisol secretion is increased in fishes transferred either from fresh water to seawater or from seawater to fresh water. Cortisol promotes branchial excretion of monovalent ions, Na^+ and Cl^-, in the freshwater fish adapting to seawater and promotes branchial uptake of monovalent ions in the seawater fish adapting to freshwater. In both freshwater and seawater teleosts, cortisol increases the number of gill chloride cells and Na^+–K^+–ATPase activity of gills, intestine and kidneys (Hazon and Balment, 1998). Daily injections of cortisol were found to increase the abundance of branchial Na^+-K^+-ATPase subunit mRNAs in *Sparus sarba* acclimated in both seawater and hypo-osmotic conditions (Deane *et al.*, 1999b). Significant increases in body influxes of Na^+ and Cl^- and also the number and individual apical surface area of gill chloride cells in freshwater rainbow trout (*Salmo gairdneri*) were observed following daily intramuscular injection of cortisol for 10 days (Laurent and Perry, 1990). McCormick (1990) has demonstrated that cortisol stimulated differentiation of chloride cells in opercular membrane of freshwater tilapia directly by increasing density and height of chloride cells and Na^+–K^+–ATPase activity. In a study on

Atlantic salmon, administration of cortisol in combination with growth hormone was found to have a synergistic effect on gill Na^+-K^+-ATPase activity (McCormick, 1996). Furthermore, cortisol was shown to increase the rate of fluid absorption by the posterior intestine in Atlantic salmon during parr-smolt transformation (Veillette *et al.*, 1995).

1.4. Salinity adaptation in teleosts

Salinity adaptation by euryhaline teleosts involves a lot of physiological changes. For example, plasma osmolarity and concentrations of Na^+ and Cl^- elevate temporarily following transfer from fresh water to seawater. For instance, plasma concentrations of Na^+ and Cl^- were shown to elevate in Mozambique tilapia (*Oreochromis mossambicus*) 1 day after transfer from fresh water to seawater (Morgan *et al.*, 1997). In a study on the euryhaline milkfish (*Chanos chanos*), the plasma osmotic concentrations were highest in fish acclimated to hyper-osmotic salinity of 55 ppt and lowest in those in hypo-osmotic salinity of 15 ppt (Swanson, 1998).

Likewise, the gill Na^+-K^+-ATPase activity and the number of branchial chloride cells usually increase in seawater. In a study on the euryhaline killifish *Fundulus heteroclitus* and parr and presmolt of Atlantic salmon (*Salmo salar*), gill Na^+-K^+-ATPase activity of both fish was demonstrated to increase after the transfer from low salinity of 0.1 ppt to high salinity of 25-35 ppt (Mancera and McCormick, 2000). Also, branchial Na^+-K^+-ATPase activity of a marine teleost, silver sea bream (*Sparus sarba*) was found to decrease after hypo-osmotic adaptation at 6 ppt for 21 days (Kelly and Woo, 1999). Birt and Green (1993) have reported that gill chloride cells increased in size in Atlantic salmon exposed to seawater and decreased in size in fish held in fresh water. On the other hand, alternation of branchial chloride cell morphology is also critical for euryhalinity. For example, the euryhaline killifish, the tight junction between adjacent chloride cells becomes shallow following seawater adaptation and assumes importance for it in secreting NaCl. On the contrary, in fresh

water, the tight junction between adjacent chloride cells is mostly deep as the branchial ionic permeability of freshwater-adapted fish is low (Karnaky, 1998).

Generally, during transfer from fresh water to seawater, plasma levels of cortisol and growth hormone would elevate. Higher cortisol release and a marked hyperplasia of the interrenal cells were observed in the seawater-adapted tilapia (Balm *et al.*, 1995). On the contrary, a significant decrease in plasma levels of growth hormone was observed in the teleost tilapia after transfer from seawater to fresh water (Yada *et al.*, 1994). In a study on juvenile Atlantic salmon, one or three injections of growth hormone significantly increased salinity tolerance as judged by lower plasma osmolality and Na^+ and higher muscle moisture content after transfer from 12 ppt to 34 ppt seawater (McCormick, 1996). Although treatment with growth hormone failed to increase gill Na^+-K^+-ATPase activity, it prevented the decreases in gill Na^+-K^+-ATPase activity that occurred in controls following transfer to 34 ppt seawater. Injections of cortisol also increased both gill Na^+-K^+-ATPase activity and salinity tolerance by lowering the plasma concentrations of Na^+. Combination of cortisol and growth hormone resulted in gill Na^+-K^+-ATPase activity significantly greater than with either hormone alone. The results indicated that both growth hormone and cortisol increased salinity tolerance and gill Na^+-K^+-ATPase activity of Atlantic salmon and the two hormones together acted synergistically (McCormick, 1996). Growth hormone acts in synergism with cortisol by increasing the number of gill glucocorticoid receptors in juvenile coho salmon (Shrimpton *et al.*, 1995).

Chapter 2:

Effect of *in vitro* thermal shock on HSP70 expression in whole blood of *Sparus sarba*

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2.1. Introduction

Thermal stress is the problem that all aquatic ectothermic animals including teleosts must face. Enhanced synthesis of heat shock proteins (HSPs) is one of the most important cellular stress responses that protect their cells from thermal stress. This set of intracellular proteins is termed heat shock proteins because they confer the cells inducible thermotolerance. Heat shock proteins can be further divided into six families according to their molecular weights. Among them, upregulation of HSP70 by high temperatures is the most prominent. Four members of HSP70 family have been identified and hsp70 (also referred to as Hsp72) is the well-known member for being inducible by heat shock. During heat shock, heat shock transcription factor 1 (HSF1) is activated and binds to the heat shock element (HSE) to initiate transcription of HSP70 genes. HSP70 prevents aggregation of proteins through binding to their hydrophobic surfaces exposed by heat-induced denaturation and assists refolding of denatured proteins (Welch *et al.*, 1998).

Heat shock has been reported to induce piscine HSP70 expression *in vitro*. Heat shock at 25°C induced *in vitro* synthesis of HSP70 in red blood cells of a coldwater-adapted species, the rainbow trout (*Oncorhynchus mykiss*). There are several advantages in choosing whole blood as an *in vitro* system to study HSP70 expression. First of all, blood is easily collected and experimentally manipulated. Likewise, blood is a non-terminal means for assessing the impact of environmental

stresses on fish. Furthermore, the main component of whole blood is red blood cells (RBCs). Unlike enucleated mammalian red blood cells, the fish RBCs are nucleated and are capable of protein synthesis, thus providing a useful model for studying HSP70 expression *in vitro* (Currie and Tufts, 1997).

The objectives of this experiment were to establish whether blood HSP70 expression of *Sparus sarba* could be used as a bioindicator of thermal stress and to study the HSP70 induction profile in whole blood of *Sparus sarba* exposed to thermal shock *in vitro*. In this *in vitro* experiment, whole blood of *Sparus sarba* was exposed to four different thermal shocks: heat shock (27°C), severe heat shock (32°C), cold shock (12°C) and the control temperature of 19°C. In order to quantify the blood HSP70 expression in silver sea bream, an indirect ELISA was developed. The results of this study support the notion that both heat and cold stress are potent stimuli for HSP70 upregulation.

2.2. Materials and methods

2.2.1. Overall experimental design

In this experiment, blood drawn from unstressed silver sea bream was exposed to 2 hours of either heat shock or cold shock. All the soluble proteins of the blood were extracted and the HSP70 levels were quantified by indirect enzyme-linked immunosorbent assay (ELISA).

2.2.2. Experimental fish

Sparus sarba, silver or gold-line sea bream, weighing from 68 to 147g, were obtained from local sea cages and transferred to aquaria equipped with seawater recirculation in the Marine Science Laboratory (MSL). Fish were acclimated to these conditions for at least 2 weeks prior to experiment. During the acclimation period, the temperature of the water was kept at 18-20°C, salinity at 33-34 ppt, and the fish were fed with a pellet diet formulated according to Woo and Kelly (1995).

2.2.3. Blood sampling and preparation

1-2.5ml of blood was taken from the caudal vessels by syringes. The syringes were rinsed with 0.1% heparin (sodium salt – from porcine intestinal mucosa; Sigma) in 0.85% saline solution in advance to prevent clotting. To minimize stress to the fish,

the whole process of blood sampling was finished within 3 minutes. Blood was put into a 1.5ml eppendorf tubes containing 0.1ml 0.1% heparin in 0.85% saline solution. The tubes were shaken very gently. The heparinized blood was then centrifuged at 10,000 rpm for 10 minutes. The plasma was discarded and the pellets were suspended and washed in 1ml Dulbecco's Modified Eagle Medium (DMEM; GibcoBRL). The resultant suspensions were centrifuged at 10,000 rpm for 10 minutes and pelleted. The pellets were resuspended in 1ml fresh DMEM for subsequent experiments.

2.2.4. Thermal stress regimes

Suspensions of blood pellets were divided into four groups at random. The first group was a control. Since this experiment was performed in winter and the water temperature on the day of experiment was 19°C, the suspensions of this group were incubated at a temperature of 19°C and were designated as the controls. The suspensions in the second group were incubated at 27°C (heat shock) and the third group was exposed to 32°C (severe heat shock). The suspensions of the last group were incubated at 12°C (cold shock). The temperatures for heat shock and severe heat shock were maintained by thermocirculators and the temperature of cold shock was kept constant by a chilling / heating plate (Torrey Pines Scientific ECHOtherm^{TM} model IC20). The suspensions of blood cells in all groups were incubated for 2 hours, after which the suspensions were centrifuged at 10,000 rpm for 10 minutes and the pellets were collected.

2.2.5. Protein extraction

The blood cells in the pellets were lysed in BUS solution (2% β-mercaptoethanol, 10M urea and 1% sodium dodecyl sulfate / SDS) and mixed by vortex. The samples were incubated above 70°C for at least 10 minutes to denature the proteases. The cells of the samples were then further disrupted by sonication for 10 minutes. The samples were centrifuged at 10,000 rpm for 10 minutes and finally the supernatants containing the soluble proteins were collected. All the samples were stored in freezer at -20°C for later analysis.

2.2.6. Protein quantification

The protein-dye binding method of Bradford (1976) was used to quantify the total soluble protein concentrations of the supernatants. First of all, 20µl of the supernatants was hydrolyzed in 80 µl 0.1M NaOH at room temperature for 3 hours. 20µl of the mixture was mixed with 1ml dye reagent of Bradford. To prepare 1L dye reagent of Bradford, 0.4g Brilliant Blue G-250 was first dissolved in 50ml ethanol (96%). Next, 100ml phosphoric acid H_3PO_4 (85%) was added to this solution. Finally, distilled water was added to 1000ml and the reagent was filtered. The absorbance of the samples was read by spectrophotometer (Milton Roy Spectronic 3000 Array) at 595nm. The total soluble protein concentrations of the samples were quantified by calibrating with several dilutions of protein standard solutions of bovine serum albumin (BSA; Sigma) with concentration between 0 and 1 mg/ml. As the standard curve becomes non-liner for protein concentrations higher than 1.4mg/ml,

sample supernatants of high protein concentrations were diluted prior to assay.

2.2.7. Indirect enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was developed for the quantification of HSP70. 10μg of total soluble protein of each sample and BUS solution was added so that a known volume of 5μl was in all tubes. The mixture was then diluted in 195μl coating buffer (0.01M Na_2CO_3, 0.01M $NaHCO_3$, pH 9.6). 50μl of each of these sample mixtures was pipetted to the flat-bottomed wells of 96-well polystyrene microtitre plate (Coaster). The plate is a solid phase to which reagent is attached passively.

A standard curve was made by a serial two-fold dilution of HSP70 (from bovine brain; Sigma) and triplicated in three columns of the plate. Bovine HSP70 solution was diluted from concentration of 0.05μg/μl to 0.781ng/μl with the same volume of BUS solution. Then, 2μl of each of the seven bovine HSP70 solutions was mixed with 3μl BUS solution and diluted in 195μl coating buffer. 50μl were added accordingly to the wells of the three columns except the wells in the last row. In this way, bovine HSP70 concentrations in the wells range from 0.0117ng/μl to 0.5ng/μl (= 0.05μg/μl × 2μl / 200μl). The blank was set by mixing 5μl BUS solution and 195μl coating buffer only. 50μl was added to the wells of the last row. The plate was incubated at 4°C in a refrigerator for 24 hours without covering the plate.

The wells were washed to separate bound and free reagents. The plate was washed six times by flushing the wells with 250μl washing buffer (0.4% NaCl, 0.1%

KCl, 0.094% $Na_2HPO_4 \cdot 2H_2O$ and 0.1% KH_2PO_4) using a Digital Multichannel Finnpipette (Labsystems). The flushing process effectively removed all free reagents, leaving the HSP70 being bound to the polystyrene. The plate was then blotted to eliminate the majority of residual wash buffer by turning the plates with the well openings down onto a piece of tissue paper and gently tapping the plate against the paper.

200µl blocking buffer (5% skimmed milk powder, 0.01M PBS, pH 7.4) was added to all wells to prevent non-specific absorption of proteins from samples. The plate was then incubated at 37°C for 2 hours.

The plate was washed 6 times in washing buffer and blot-dried on tissue paper as described above. 50µl of monoclonal (mouse) anti-heat shock protein 70 antibody (IgG) (ABR) diluted 1:10,000 in PBS-T was added to all wells. The plate was put on a platform shaker (Stuart Scientific STR6) and shaken gently. After incubation at room temperature for 1 hour, the plate was washed 6 times in washing buffer and blot-dried. 100µl anti-mouse Ig, horseradish peroxidase linked whole antibody (from sheep) general purpose reagent (GPR) (Amersham Life Science) diluted 1:10,000 in PBS-T was pipetted into each well. Following incubation on the platform shaker at room temperature for 1 hour, the plate was washed 6 times in washing buffer and blot-dried. One 3,3',5,5'-tetramethylbenzidine (TMB) tablet (Sigma) was dissolved in 10ml 0.05M phosphate-citrate buffer (0.2M Na_2HPO_4, 0.1M citric acid and 0.03% sodium perborate) at pH 5. 200µl of TMB substrate was added to the wells and the plate was incubated on the platform shaker at room temperature for 15 minutes. The colour development was stopped by addition of 50µl 2M H_2SO_4. Lastly, the HSP70

was quantified by reading the plate with microplate spectrophotometer (SPECTRAmax^{TM} 250) at wavelength of 450nm.

2.2.8. Protein gel electrophoresis and immunoblotting (Western blotting)

Representative blots were prepared by protein gel electrophoresis and then immunoblotting. Laemmli one-dimensional SDS-PAGE was firstly applied to resolve proteins of different molecular sizes. A 4% stacking and a 12% separating SDS polyacrylamide gel were used. 20μg of total soluble protein of samples mixed with loading buffer (1.2% Tris base, 10% SDS, 10% β-mercaptoethanol, 20% glycerol and 0.1% bromophenol blue, pH6.8) were loaded into wells formed by the 4% stacking gel and electrophoresed by a Bio-Rad mini gel kit for 1 hour at 140V.

Western blotting was then conducted. The resolved proteins were transferred from the separating gel to a nitrocellulose membrane (GibcoBRL) by an electrotransfer cell (Bio-Rad) for 1 hr at 160V. The membrane was then air-dried for a few minutes and blocked in 0.01M phosphate-buffered saline (PBS, pH7.4) containing 0.05% Tween-20 (PBS-T) with 3% skimmed milk powder overnight at 4°C. The membrane was then incubated with two layers of specific antibodies. The primary antibody was monoclonal (mouse) anti-heat shock protein 70 antibody (IgG) (ABR) diluted 1:20,000 in PBS-T and the secondary antibody was anti-mouse Ig, horseradish peroxidase linked whole antibody (from sheep) GPR (Amersham Life Science) diluted 1:20,000 in PBS-T. In between the incubations, the membrane was washed for 15 minutes three times in PBS-T. Finally, the HSP70 bands of samples were visualized by adding a

detection reagent of an ECL development system (Amersham International plc) onto the membrane and incubating it in lumi-imager F1 (Roche) for 1 minute.

2.2.9. Statistical analysis

In the indirect ELISA, absorbance values were plotted against bovine standard HSP70 protein (ng/μl) or sample protein (ng/μl) and the relationship quantified using linear regression analysis. The significance of the regression line was estimated by calculating the coefficient of determination (r^2). Parallelism, between the standard curve of bovine HSP70 and the titration curve of serially diluted whole blood protein, were assessed by comparing the slopes of the regression lines using t test (Zar, 1984). The HSP70 values are presented as means ± standard error (SEM) and were subjected to a one-way ANOVA to test for significance. Subsequent significance between groups was delineated by a Student-Newman-Kuels test (SigmaStat statistical software, Jandel Scientific).

2.3. Results

2.3.1. Validation of indirect ELISA

The validity of the indirect ELISA developed for quantifying the blood HSP70 expression in *Sparus sarba* was ascertained using serial dilutions of bovine standards and samples. Comparison of the slope of the titration curve of serially diluted whole blood protein and that of the standard curve of bovine HSP70 yielded no significant difference ($t=0.428$; $t_{0.05\,(2),5}=2.571$, $p>0.5$) (Figs. 2.1., 2.2. and 2.3.). Therefore, the standard curve of bovine HSP70 and the titration curve of serially diluted whole blood protein displayed parallelism and the indirect ELISA for sea bream whole blood HSP70 has been developed successfully.

2.3.2. Effect of *in vitro* thermal shock on HSP70 expression in whole blood of *Sparus sarba*

HSP70 levels of whole blood were upregulated after exposure for 2 hours to heat (27°C), severe heat (32°C) or cold (12°C) stress *in vitro* (Figs. 2.4. and 2.5.). The mean blood HSP70 level of *Sparus sarba* incubated at the control temperature, 19°C, was 9.39 ± 0.62 ng/μg total soluble protein. The HSP70 level at 27°C was higher than that at the control temperature by 263%. The maximal HSP70 synthesis was at 32°C. The HSP70 level at 32°C was upregulated by 369% against control. On the other hand, 2 hours cold shock at 12°C also significantly increased the levels of HSP70 in whole blood. However, there was no significant difference between the HSP70 levels at 12°C and 27°C.

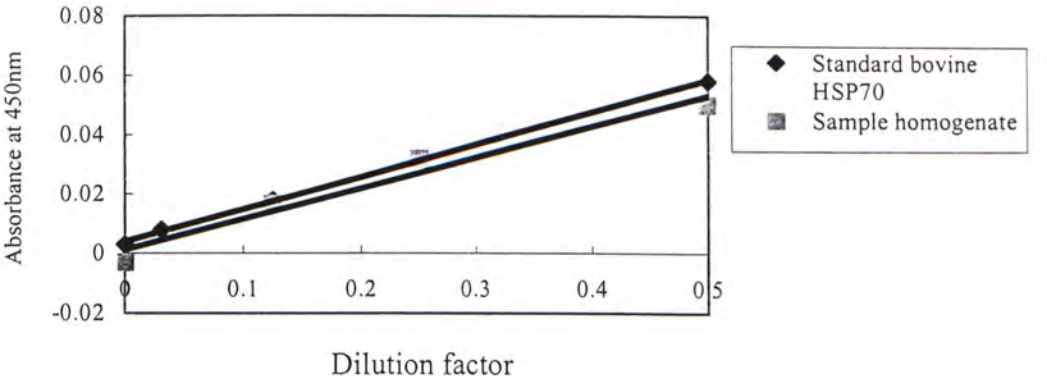

Figure 2.1. Parallelism between titration curve of serially diluted whole blood protein of *Sparus sarba* and standard curve of bovine HSP70.

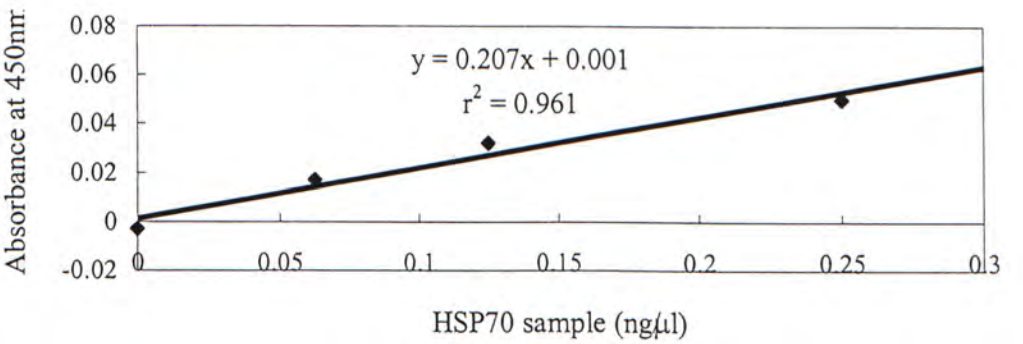

Figure 2.2. Titration curve of serially diluted whole blood protein of *Sparus sarba*.

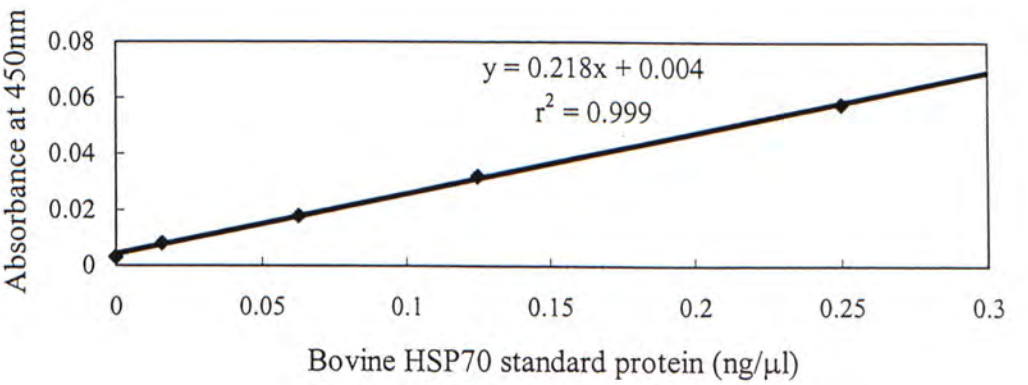

Figure 2.3. Standard curve of bovine HSP70.

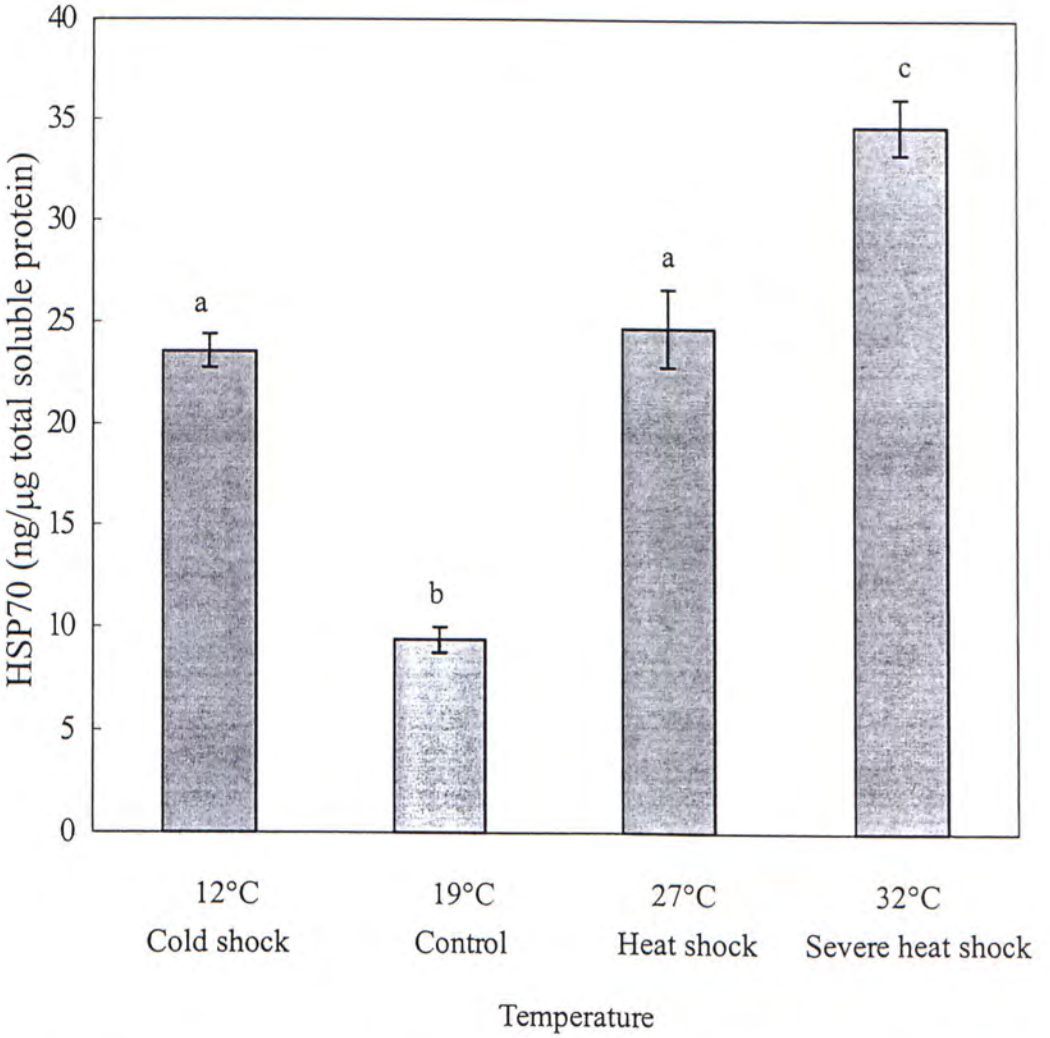

Figure 2.4. Effect of temperature on HSP70 expression in the whole blood of *Sparus sarba* as determined by indirect ELISA. Values are means ± S.E.M. HSP70 levels (n=5). Values with different alphabets are significantly different from each other (P<0.05), Student-Newman-Kuels Multiple Comparison.

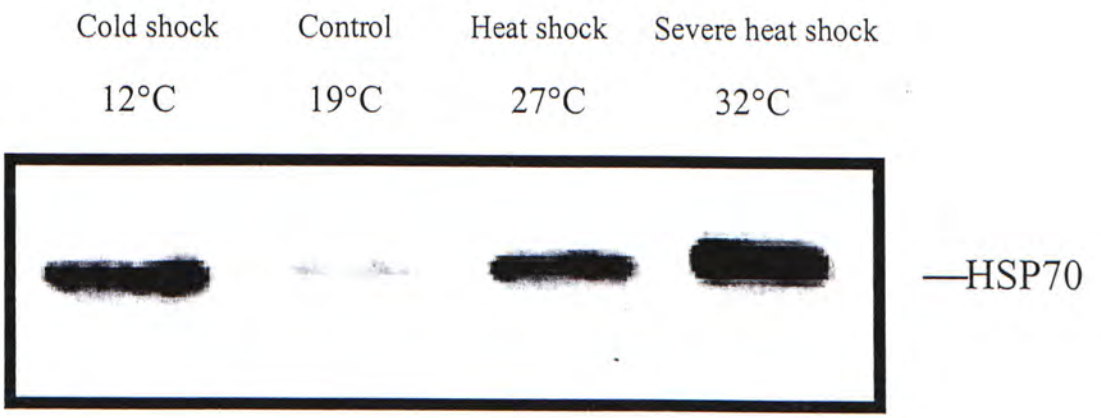

Figure 2.5. Representative western blot analysis of HSP70 in *Sparus sarba* whole blood at 12°C, 19°C, 27°C and 32°C.

2.4. Discussion

In the present study, the mean value of blood HSP70 of *Sparus sarba* incubated at the control temperature, 19°C, was 9.39 ± 0.62 ng/μg total soluble protein. The values of hepatic HSP70 of *Sparus sarba* at ambient temperature of 25°C have been assessed (Deane *et al.*, 1999a, 2000a). The hepatic HSP70 ranged from 5ng/μg to 12ng/μg. The control values of blood HSP70 of the present study appeared to be in line with the values of hepatic HSP70 reported in these studies.

The results indicated that HSP70 was induced in whole blood of *Sparus sarba* after *in vitro* exposure to 27°C and 32°C from a temperature of 19°C. The temperature at which HSP70 synthesis was induced was between 19 and 27°C and the synthesis continued to increase up to 32°C. *In vitro* exposure to heat shock also induces HSP70 synthesis in other tissues of fish. In one study, the HSP70 levels in primary cultures of hepatocytes, gill epththelial cells and fibroblast-like RTG-2 cells of rainbow trout were found to elevate after heat shock at 26°C (Airaksinen *et al.*, 1998). The HSP70 induction by heat shock in whole blood is quite similar to that observed in an *in vivo* study on hepatic tissue of *Sparus sarba* (Deane *et al.*, 1999a, 2000a). However, in these experiments, the control temperature was 25°C and the temperature of heat shock was 32°C, that was 7°C higher than the control. From one study, 32°C was found to be the ultimate upper incipient lethal temperature of a teleost fish that is closely related to *Sparus sarba*, red sea bream (*Chrysophyrs major*) (Woo and Fung, 1980). In another study on red sea bream, compared to those adapted to 10, 20, 25°C for 21 days, the fish acclimated to 30°C for the same number of days was shown to have significantly elevated plasma osmolality and concentration of Na^+, lower liver

total protein and muscle total lipid levels but higher liver and muscle glycogen storage (Woo, 1990). The results implied that high temperature enhanced protein and lipid catabolism and glycogen deposition. Ionic and metabolic disturbances occurred at 30°C. Therefore, these studies suggested a temperature of 32°C might be very stressful to *Sparus sarba* that also prefers cooler waters, and the high levels of blood HSP70 at 32°C from the present study might also indicate an extreme stress state.

Also, induction of HSP70 expression in fish blood by *in vitro* exposure to heat shock has been reported. Heat shock at 25°C induced *in vitro* synthesis of HSP70 in red blood cells of a coldwater-adapted species, the rainbow trout (*Oncorhynchus mykiss*) (Currie and Tufts, 1997). In this experiment, the control temperature was taken as 10°C. This temperature was the temperature at which the fish was acclimated. No HSP70 upregulation was observed when cells were exposed to 15°C and 20°C but marked induction was evident at 25°C. The author suggested that HSP70 synthesis was induced was between 20 and 25°C. Thus whether HSP70 is induced or not at a particular temperature depends on the acclimation temperature of the fish (Currie *et al.*, 2000). Working with an *in vitro* rainbow trout blood preparation, Currie *et al.* (2000) found that for fish acclimated to warm water of 17°C, marked HSP70 mRNA induction was observed at 20°C. The ambient temperature was increased for only 3°C. However, for fish acclimated to cold water of 5°C, no significant increase in HSP70 was detected until the temperature was increased to 25°C. A considerably larger temperature difference (20°C) was required to elicit the same response. The threshold inductio n temperature of the heat-shock response may be related to the expression of heat-shock genes, which in turn, is determined by the thermal stabilities of cellular proteins (Dietz and Somero, 1993). Apart from

acclimation temperature, the induction profile of HSP70 is also species-specific and tissue specific (Dietz and Somero, 1993). Wide variation in average threshold HSP70 induction temperature (~ 8°C) was found among four species of marine teleost fishes, buffalo sculpin (*Enophrys bison*), speckled sanddab (*Citharichthys stigmaeus*), English sole (*Parophrys vetulus*) and Pacific staghorn sculpin (*Leptocottus armatus*), acclimatized to a common temperature (10°C). Variation in HSP70 induction temperatures was also found among tissues including brain, gill and liver of a species, but to a lesser degree. Also, the temperature at which maximal HSP70 induction can be observed appears to be species-specific. In buffalo sculpin, the average threshold HSP70 induction temperature of brain, gill and liver was 20.3°C but the HSP70 levels peaked at or below 24°C. Further increase in exposure temperature brought the HSP70 synthesis down from the maximum. On the contrary, HSP70 synthesis in red blood cells of eurythermal killifish *Fundulus heteroclitus* was induced by heat shock at 32.5°C and the HSP70 mRNA levels has shown to increase further after heat shock at 40°C (Koban *et al.*, 1991). In this respect, the HSP70 induction profile of whole blood in *Sparus sarba* is similar to that of red blood cells in *Fundulus heteroclitus*.

The results of the present study also showed that *in vitro* cold shock at 12°C induced the HSP70 expression in whole blood of *Sparus sarba*. However, *in vivo* cold shock at 16°C was reported to have no effect on hepatic HSP70 expression in *Sparus sarba* (Deane *et al.*, 1999a, 2000a). There are not many works to address the induction of HSP70 synthesis in fish by cold shock. In one study, cold acclimation at 4°C was shown to induce the synthesis of a 70kDa protein in rainbow trout cell line RTG-2 (Yamashita *et al.*, 1996). It was thought that the induction was a metabolic compensation for the delay in cell cycling due to low temperatures. It also suggested

that the 70kDa protein might be directly involved in the cold acclimation. In mammals, cold shock was reported to significantly elevate HSP70 levels in terms of both protein and mRNA in primary cultures of neonatal rat cardiomyocytes (Laios *et al.*, 1997). Cold shock can also induce HSP70 expression in flesh fly (*Sarcophaga crassipalpis*). For example, in one experiment, HSP70 was expressed in brain and integument of flesh fly during recovery from cold shocks of –10°C or –18°C (Joplin *et al.*, 1990).

2.5. Conclusion

A sensitive indirect ELISA has been developed for the quantification of blood HSP70 protein of *Sparus sarba*. The ELISA has been validated using serial dilution of bovine HSP70 as the standard protein and has been found to provide an accurate quantification of blood HSP70 from 0.0117ng/μl to 0.5ng/μl. The HSP70 levels of whole blood of *Sparus sarba* were upregulated by *in vitro* exposure to thermal stress for 2 hours from a control temperature of 19°C. The temperature at which HSP70 synthesis was induced was between 19°C and 27°C. The maximal HSP70 induction was at 32°C. On the other hand, 2-hour cold shock at 12°C also significantly increased the levels of HSP70 in whole blood. The results supported the notion that both heat and cold stress were potent stimuli for blood HSP70 upregulation.

Chapter 3:

Effects of hormones on HSP70 expression in whole blood of *Sparus sarba in vitro*

Chapter 3: Effects of hormones on HSP70 expression in whole blood of *Sparus sarba in vitro*

3.1. Introduction

Although the biology of heat shock proteins has been well studied, the endocrine regulation of HSP70 expression in fish remains unclear. Therefore, to investigate HSP70 expression in whole blood of silver sea bream (*Sparus sarba*) after *in vitro* treatment with different hormones is the most important objective of the present study. Four hormones have been chosen. They are cortisol, recombinant bream growth hormone (rbGH), recombinant bream insulin-like growth factor-I (rbIGF-I) and ovine prolactin (oPRL). Cortisol is a classic stress hormone and plasma cortisol is used as a primary stress response parameter (Wendelaar Bonga, 1997). HSP70 is a stress response protein. There may be a relationship between them. The growth-promoting effects of growth hormone are well-known and IGF-I is a factor to mediate the growth-promoting effects of growth hormone. Prolactin is a versatile protein hormone and belongs to the same hormone family of growth hormone. Prolactin and growth hormone are similar in structure and amino acid sequence. Although stress-induced growth suppression was suggested, the importance of growth hormone, IGF-I and prolactin during stress has not yet been established in fish. In this study, how these hormones are related to stress was examined by using HSP70 expression as a stress indicator (Ryan and Hightower, 1996).

Apart from Deane *et al.* (1999a), as yet no other works have addressed the direct effects of hormones on HSP70 levels in fish. In this *in vivo* study, cortisol was

found to have no effect on hepatic HSP70 expression of *Sparus sarba*. In contrast, both rbGH and oPRL caused a significant decrease in hepatic HSP70 levels. Similar results were found in the present *in vitro* experiment. In the present study, cortisol did not affect blood HSP70 expression in *Sparus sarba*. On the contrary, rbGH, rbIGF-I and oPRL were all shown to reduce the HSP70 levels significantly. Since HSP70 expression can be used as a stress indicator, the present study will report (a) conditions of enhanced growth hormone, IGF-I and prolactin status and reduced HSP70 might be related to a state of reduced stress in fish, and (b) the classic stress hormone cortisol appeared to be unrelated to HSP70 expression.

3.2. Materials and methods

3.2.1. Overall experimental design and experimental fish

Silver sea bream (*Sparus sarba*), weighing between 52g and 208g, kept unstressed in normal seawater for at least 2 weeks were sacrificed and blood was collected from the caudal vessels by heparinized syringes. The methods of blood sampling and blood preparation were exactly the same as those described in Materials and Methods of Chapter 3. The blood pellets were randomly divided into three groups. The first group was the saline control group. Blood pellets of this group were incubated at ambient temperature of 19°C for 2 hours in fresh DMEM (GibcoBRL) into which 0.85% saline was added. This group served as the control for the second group, hormone treatment group. Pellets of this group were subdivided into several groups. Blood pellets of each subgroup were incubated at ambient temperature for 2 hours in fresh DMEM into which a hormone solution of a particular concentration was added. The last group was the untreated group. Pellets of this group were immediately frozen in liquid nitrogen and stored in deep freezer at -70°C. This group served as a control for the saline control group. All the soluble proteins of the blood were extracted and the HSP70 levels in the blood of all the treatment groups were assessed by indirect enzyme-linked immunosorbent assay (ELISA) as outlined in Chapter 3.

3.2.2. Hormone treatments

The blood of *Sparus sarba* was incubated with four different hormones. They are cortisol (hydrocortisone; Solu-Cortef; Upjohn, Belgium), recombinant bream growth hormone (rbGH; GroPep Pty Ltd, Australia), recombinant bream insulin-like factor-I (rbIGF-I; GroPep Pty Ltd, Australia) and ovine prolactin (oPRL; from sheep pituitary glands; Sigma, USA). For the cortisol treatment, the hormone treatment group was divided into three subgroups. Cortisol was dissolved firstly in sterilized 0.85% saline and then diluted in fresh DMEM to three final concentrations (10ng/ml, 50ng/ml and 100ng/ml). These concentrations of cortisol were chosen for study because the physiological serum level of cortisol in *Sparus sarba* ranges between 8 and 14ng/ml (Deane *et al.*, 2000b, 2001b) and 50ng/ml and 100ng/ml are probably the serum cortisol levels in a stressed fish. The blood pellets of the subgroups were incubated in these three concentrations respectively. As the physiological serum levels of growth hormone, IGF-I and prolactin of *Sparus sarba* have not been addressed in any study, therefore a wide range of doses (1ng/ml, 10ng/ml, 100ng/ml and 1000ng/ml) was used for these hormones. rbGH, rbIGF-I and oPRL were respectively dissolved in sterilized 0.85% saline and diluted in fresh DMEM to these four concentrations. The blood pellets in all treatment groups were incubated for 2 hours, after which the suspensions of blood cells were centrifuged at 10,000 rpm for 10 minutes and the pellets were collected.

3.2.3. Protein extraction and quantification, indirect ELISA, gel electrophoresis, and immunoblotting (Western blotting)

Procedures of protein extraction and quantification, indirect ELISA, gel electrophoresis and immunoblotting were exactly the same as those described in Materials and Methods in Chapter 3.

3.2.4. Statistical analysis

All data are presented as means ± standard error (SEM) and were subjected to a one-way ANOVA to test for significance. Subsequent significance between groups was delineated by a Student-Newman-Kuels test (SigmaStat statistical software, Jandel Scientific).

3.3. Results

3.3.1. Effect of cortisol on HSP70 levels in whole blood

In vitro exposure to cortisol did not affect the HSP70 levels in whole blood (Figs. 3.1. and 3.2.). There was no significant difference in HSP70 levels among between untreated, saline control, and the three groups treated with different concentrations of cortisol (10ng/ml, 50ng/ml and 100ng/ml).

3.3.2. Effect of recombinant bream growth hormone on HSP70 levels in whole blood

There was no significant difference in HSP70 levels among untreated, saline control, and the two groups treated with the two lower concentrations of recombinant bream growth hormone (rbGH) (1ng/ml and 10ng/ml) (Figs. 3.3. and 3.4.). However, when compared to the saline control group, 100ng/ml and 1000ng/ml of rbGH significantly reduced HSP70 in whole blood by 26% and 50% respectively. *In vitro* exposure to rbGH reduced HSP70 levels in whole blood in a dose-dependent manner.

3.3.3. Effect of recombinant bream insulin-like growth factor-I on HSP70 levels in whole blood

There was no significant difference in HSP70 levels among untreated, saline

control, and the two groups treated with the two lower concentrations of recombinant bream insulin-like growth factor-I (rbIGF-I) (1ng/ml and 10ng/ml) (Figs. 3.5. and 3.6.). The two higher concentrations of rbIGF-I, 100ng/ml and 1000ng/ml, however, significantly reduced HSP70 in whole blood by 28% and 48% respectively from the saline controls. *In vitro* exposure to rbIGF-I reduced Hsp70 levels in whole blood in a dose-dependent manner.

3.3.4. Effect of ovine prolactin on HSP70 levels in whole blood

No significant difference in HSP70 levels among untreated, saline control, and the group treated with the lowest concentration of ovine prolactin (oPRL), 1ng/ml, were observed (Fig. 3.7. and 3.8.). The three higher concentrations of oPRL (10ng/ml, 100ng/ml and 1000ng/ml) significantly decreased HSP70 in whole blood by 26%, 47% and 65% respectively when compared to the saline control group. *In vitro* exposure to oPRL reduced HSP70 levels in whole blood in a dose-dependent manner.

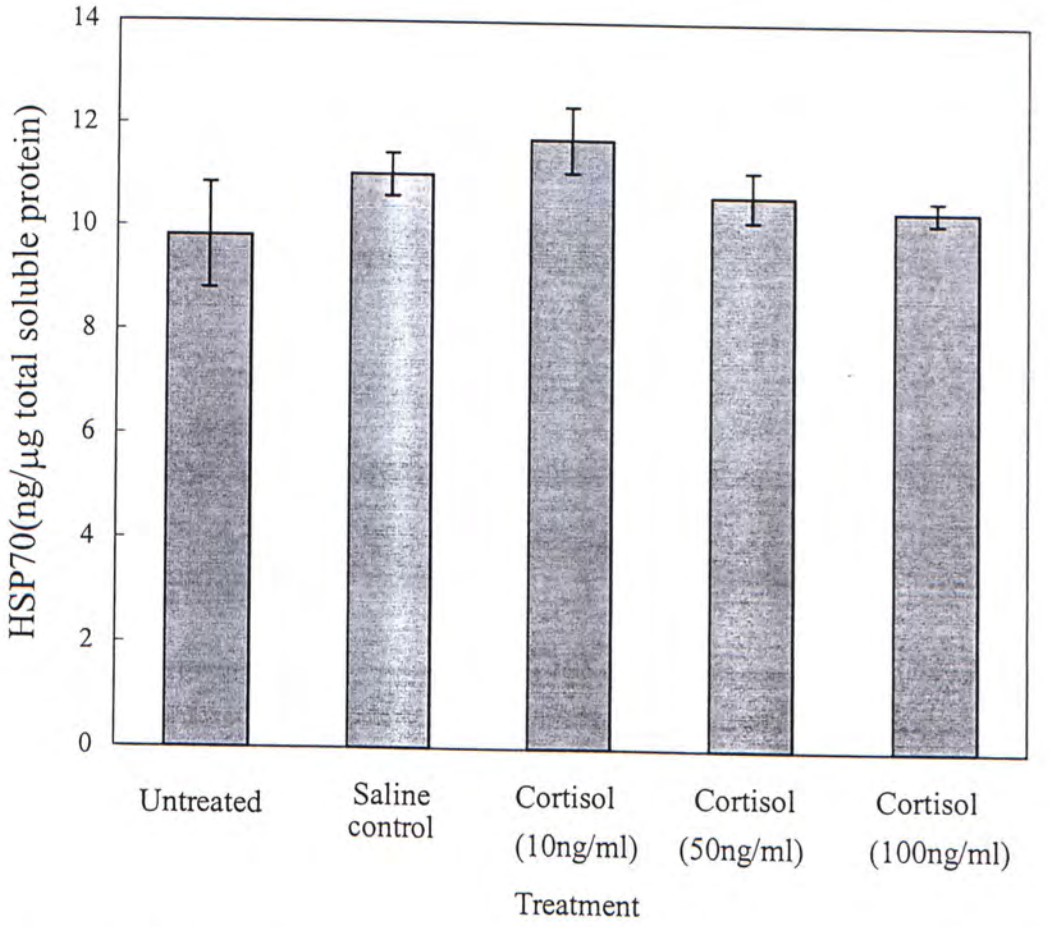

Figure 3.1. HSP70 expression in *Sparus sarba* whole blood treated with saline, cortisol (10ng/ml), cortisol (50ng/ml) and cortisol (100ng/ml) as determined by indirect ELISA. The untreated group served as a control for the saline control group which, in turn, served as the control for treated with the cortisol treatment groups. Values are means ± S.E.M. HSP70 levels (n=5).

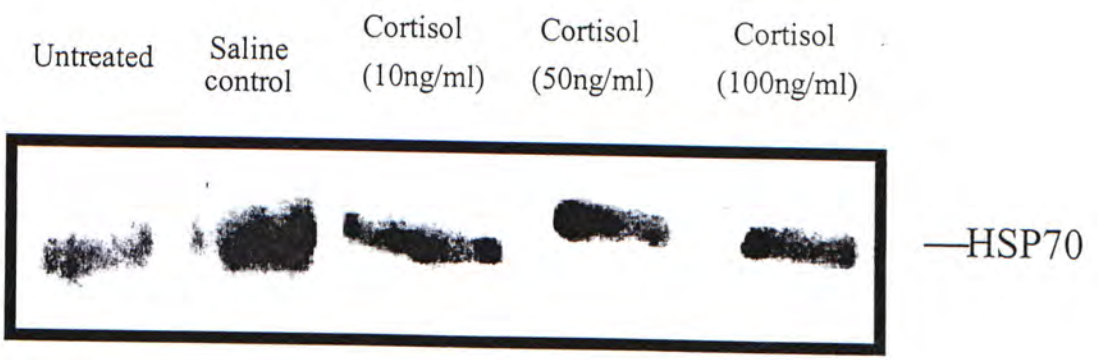

Figure 3.2. Representative western blot analysis of HSP70 in *Sparus sarba* whole blood in untreated, saline control, cortisol (10ng/ml), cortisol (50ng/ml) and cortisol (100ng/ml) groups.

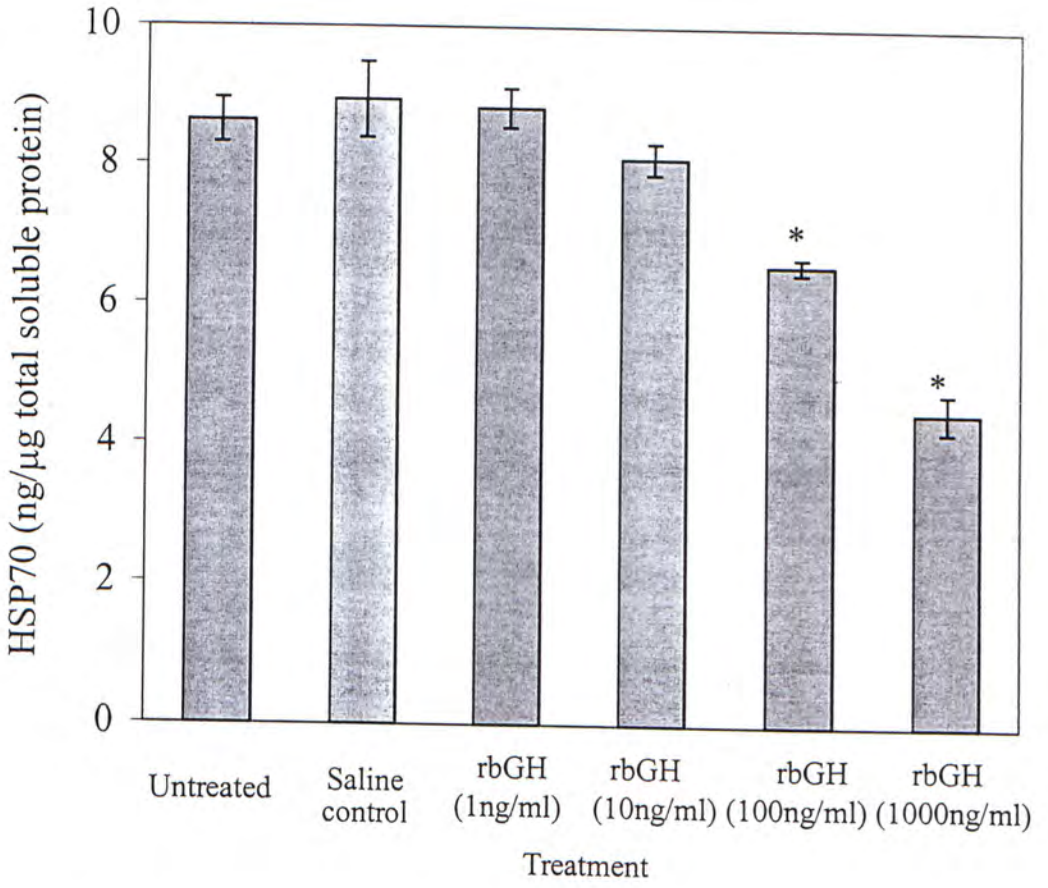

Figure 3.3. HSP70 expression in *Sparus sarba* whole blood treated with saline, rbGH (1ng/ml), rbGH (10ng/ml), rbGH (100ng/ml) and rbGH (1000ng/ml) as determined by indirect ELISA. The untreated group served as a control for the saline control group which, in turn, served as the control for rbGH treatment groups. Values are means ± S.E.M. HSP70 levels (n=7). The asterisk (*) indicates a significant difference (P<0.05) from the saline control.

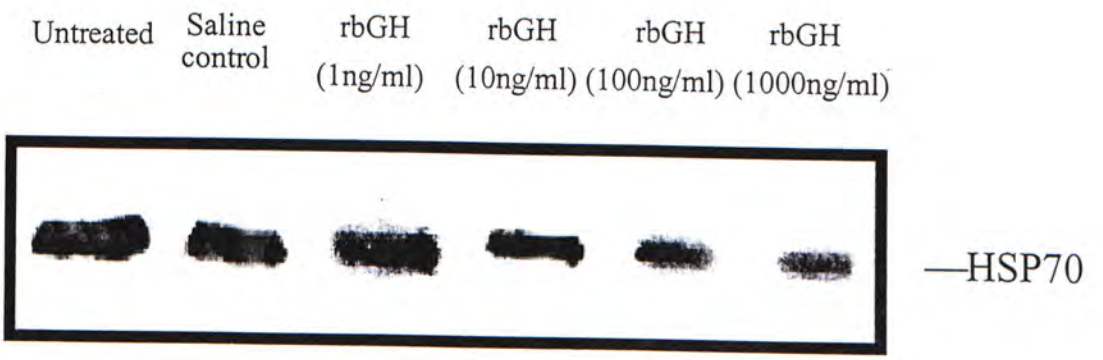

Figure 3.4. Representative western blot analysis of HSP70 in *Sparus sarba* whole blood in untreated, saline control, rbGH (1ng/ml), rbGH (10ng/ml), rbGH (100ng/ml) and rbGH (1000ng/ml) groups.

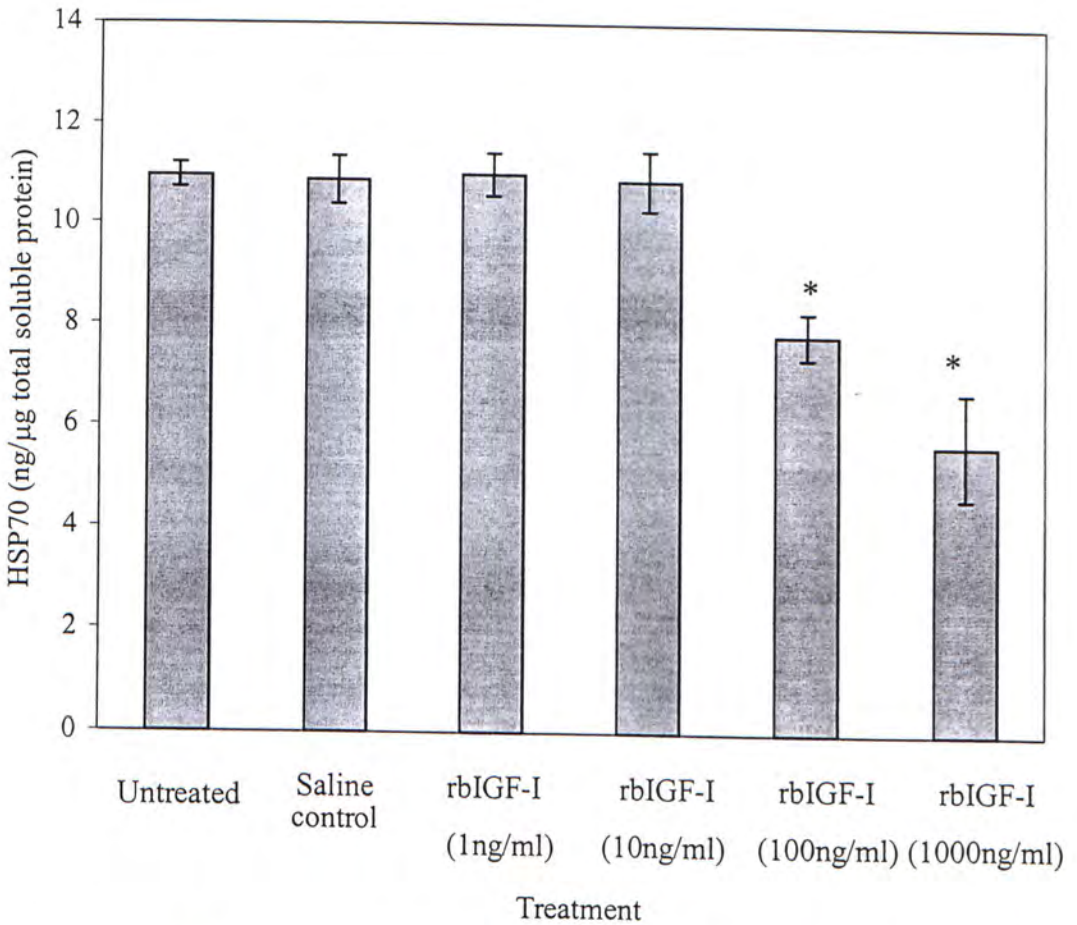

Figure 3.5. HSP70 expression in *Sparus sarba* whole blood treated with saline, rbIGF-I (1ng/ml), rbIGF-I (10ng/ml), rbIGF-I (100ng/ml) and rbIGF-I (1000ng/ml) as determined by indirect ELISA. The untreated group served as a control for the saline control group which, in turn, served as the control for rbIGF-I treatment groups. Values are means ± S.E.M. HSP70 levels (n=7). The asterisk (*) indicates a significant difference (P<0.05) from the saline control.

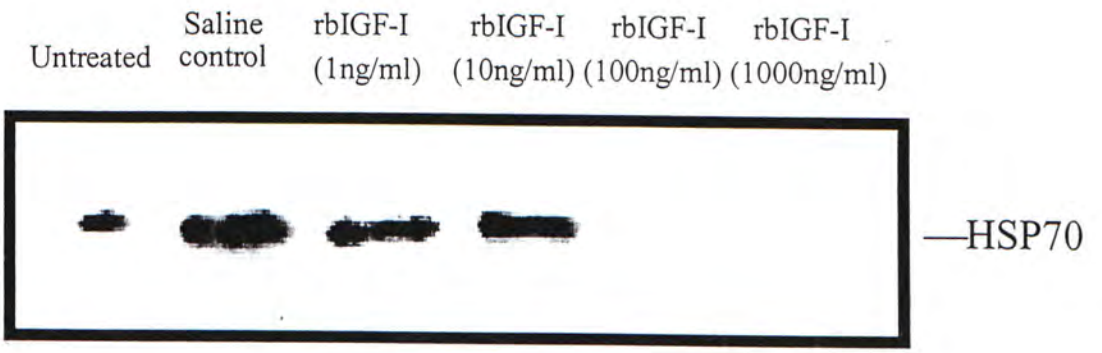

Figure 3.6. Representative western blot analysis of HSP70 in *Sparus sarba* whole blood in untreated, saline control, rbIGF-I (1ng/ml), rbIGF-I (10ng/ml), rbIGF-I (100ng/ml) and rbIGF-I (1000ng/ml) groups.

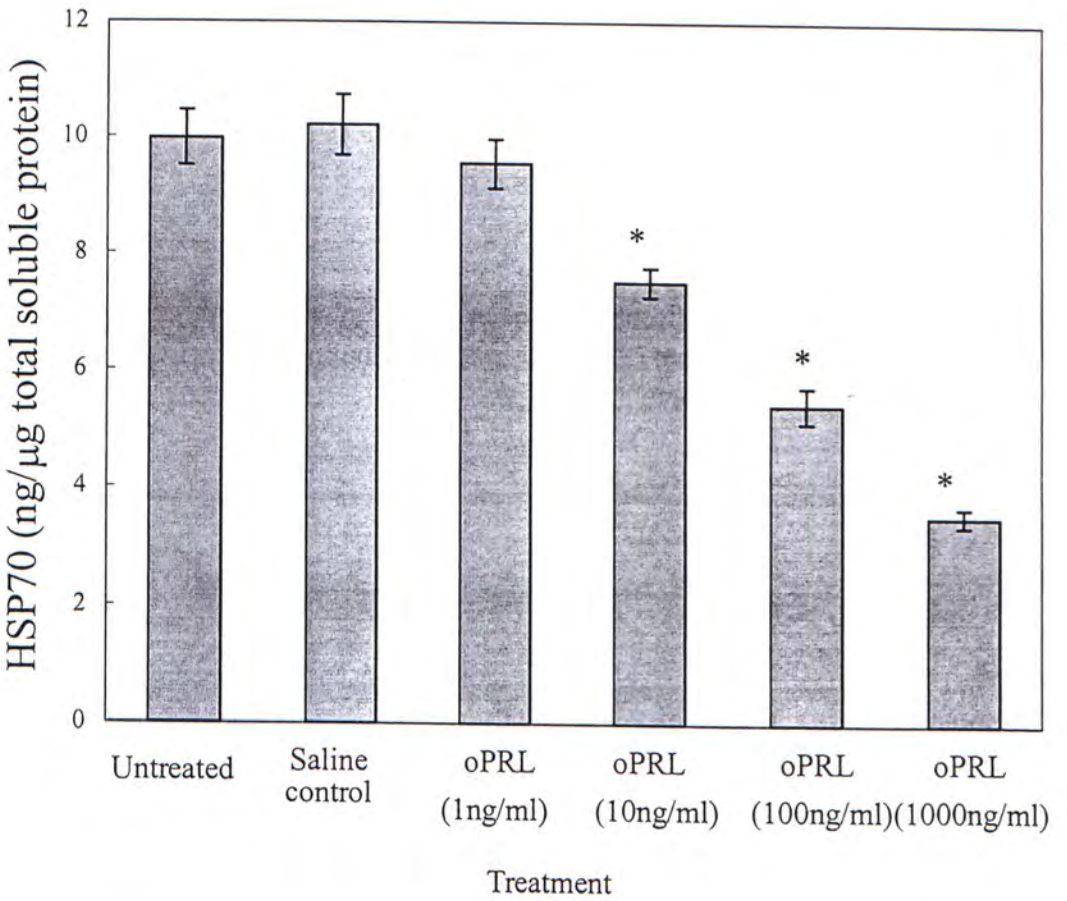

Figure 3.7. HSP70 expression in *Sparus sarba* whole blood treated with saline, oPRL (1ng/ml), oPRL (10ng/ml), oPRL (100ng/ml) and oPRL (1000ng/ml) as determined by indirect ELISA. The untreated group served as a control for the saline control group which, in turn, served as the control for oPRL treatment groups. Values are means ± S.E.M. HSP70 levels (n=7). The asterisk (*) indicates a significant difference (P<0.05) from the saline control.

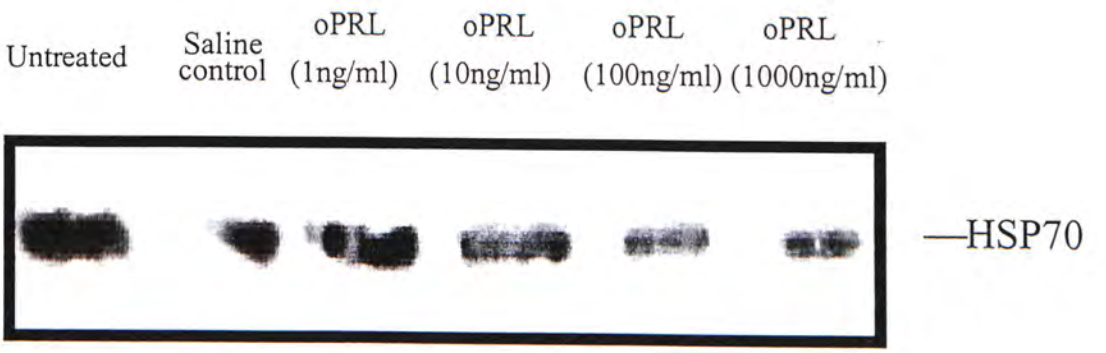

Figure 3.8. Representative western blot analysis of HSP70 in *Sparus sarba* whole blood in untreated, saline control, oPRL (1ng/ml), oPRL (10ng/ml), oPRL (100ng/ml) and oPRL (1000ng/ml) groups.

3.4. Discussion

3.4.1. Effect of cortisol on HSP70 levels in whole blood

In this study, whole blood of *Sparus sarba* was incubated with three concentrations of cortisol: 10ng/ml, 50ng/ml and 100ng/ml. The physiological serum level of cortisol in *Sparus sarba* was reported to range between 8 and 14ng/ml (Deane *et al.*, 2000b, 2001b). Stress leads to high blood cortisol levels in fish (Wendelaar Bonga, 1997). The plasma cortisol concentration in rainbow trout (*Oncorhyncus mykiss*) was demonstrated to increase from below 10ng/ml to higher than 60ng/ml at 3 hours after a 3-min handling stress (Vijayan *et al.*, 1997a). The unstressed level of plasma cortisol of golden perch (*Macquaria ambigua*) was also lower than 10ng/ml. Three hours of chronic shallow stress elevated the mean plasma cortisol level to 57ng/ml (Carragher and Rees, 1994). The unstressed plasma cortisol concentration in tilapia was about 20ng/ml and two hours of confinement stress raised the plasma cortisol concentration to over 100ng/ml (Vijayan *et al.*, 1997b). Therefore, the concentrations of 50ng/ml and 100ng/ml are probably the serum cortisol levels in a stressed fish.

In the present study, *in vitro* exposure to cortisol did not affect the HSP70 levels in whole blood. Although cortisol is a classic stress hormone and HSP70 is a cellular stress response protein, cortisol appeared to be unrelated to the HSP70 expression in whole blood of *Sparus sarba*. Daily administration of exogenous cortisol was also found to have no effect on hepatic HSP70 expression in *Sparus sarba* (Deane *et al.*, 1999a). Until recently, no other studies have investigated the direct

effect of cortisol on HSP70 expression in fish. One study reported that a 3-min handling stress increased significantly the plasma cortisol concentration but had no effect on the hepatic HSP70 levels in rainbow trout *Oncorhyncus mykiss* (Vijayan *et al.*, 1997a). In one study, how cortisol modulates HSP90 mRNA expression in primary culture of rainbow trout hepatocytes was examined. Exposure of trout hepatocytes to cortisol for 24 hours at ambient temperature did not affect HSP90 mRNA expression. However, high level of cortisol (1000ng/ml) was found to attenuate the HSP90 mRNA expression induced by heat shock. The authors suggested cortisol regulated transcription of heat shock proteins only when the levels of the classic stress hormone were high under stressful conditions (Sathiyaa *et al.*, 2001). The most clearly established relationship between cortisol and heat shock proteins is that HSP90 dimer regulates interactions between cortisol and glucocorticoid receptor (GR) by binding to GR to form an intermediate aporeceptor complex and maintain the receptor in a transcriptionally inactive state in the absence of cortisol (Scheibel and Buchner, 1998).

However, there are some works which examined the relationship between the hypothalamic-pituitary-adrenal (HPA) axis and HSP70 expression in mammals. One study suggested that the stressor like restraint stress might induce HSP70 expression by activation of HPA axis in rats (Blake *et al.*, 1991). In this experiment, the HSP70 mRNA expression in the adrenal of the hypophysectomized rats was observed not to increase in response to restraint stress. However, exogenous administration of ACTH restored the rapid induction of HSP70 mRNA expression in the adrenal cortex of these restrained hypophysectomized rats. In another study, pellets containing dexamethasone, a synthetic glucocorticoid, were implanted into the intrascapular

subcutaneous tissue of rats and then left in situ for 2 weeks (Udelsman *et al.*, 1994). The rats were then either subjected to 90 minutes of restraint stress or sacrificed immediately. The results showed that the induction of HSP70 mRNA expression in the adrenal cortical tissue of rats undergoing restraint stress was markedly attenuated by the dexamethasone. At the same time, it was demonstrated that dexamethasone also attenuated plasma ACTH level in both unrestrained and restrained rats. This study suggested that the dexamethasone-induced downregulation of HSP70 was associated with diminished ACTH secretion (Udelsman *et al.*, 1994).

3.4.2. Effect of recombinant bream growth hormone on HSP70 levels in whole blood

As the physiological serum level of growth hormone of *Sparus sarba* has not been addressed in any study, therefore a wide range of doses (1ng/ml, 10ng/ml, 100ng/ml and 1000ng/ml) was used. In one study, the plasma concentration of growth hormone of tilapia (*Oreochromis niloticus*) was shown to drop from approximately 0.8ng/ml before confinement stress to about 0.3ng/ml during the stress (Auperin *et al.*, 1997). The plasma levels of growth hormone in rainbow trout were also very low. The mean plasma growth hormone level of fish during and after 1 hour of acute handling and confinement stress was 0.66 ± 0.09ng/ml while the mean plasma growth hormone level of the unstressed controls was 1.26 ± 0.16ng/ml (Pickering *et al.*, 1991).

The physiological implication of the downregulation of HSP70 by growth

hormone in whole blood is uncertain. Daily intraperitoneal injection of rbGH was also shown to reduce hepatic HSP70 levels in *Sparus sarba* significantly (Deane *et al.*, 1999a). Like cortisol, no other studies have investigated the direct effect of growth hormone on HSP70 expression in fish and the role of growth hormone in the stress response in fish is also not clear. One team has reported that a significant decrease in plasma concentrations of growth hormone in freshwater-adapted male tilapia during confinement stress for both 1 hour and 24 hours (Auperin *et al.*, 1997). An acute handling stress followed by confinement stress also decreased the concentration of circulating growth hormone significantly in rainbow trout (Pickering *et al.*, 1991). In both cases, stress-induced growth suppression was suggested. In mammals, plasma concentrations of growth hormone were also found to decrease in lactating Holstein cows during acute thermal stress caused by elevated temperatures (McGuire *et al.*, 1991). In fact, growth hormone-induced growth enhancement was believed to be an indicator of reduced stress in fish (Wendelaar Bonga, 1997). For instance, Atlantic salmon (*Salmo salar*) parr subjected to either once daily or twice daily acute handling stresses had lower growth rates in length and weight than controls (McCormick *et al.*, 1998). On the other hand, growth hormone was reported to stimulate the non-specific immune response in fish by increasing macrophage phagocytosis (Sakai *et al.*, 1995). In one study, injection of growth hormone in juvenile *Sparus sarba* enhanced macrophage phagocytic activity in terms of both percent phagocytosis and phagocytic index of macrophages of both head-kidney and spleen (Narnaware *et al.*, 1997). Since reduced HSP70 expression could indicate a lowered stress level, the downregulation of HSP70 suggested that growth hormone might be related to a reduced stress state. However, the detailed mechanism by which growth hormone interacts with HSP70 molecules is not yet understood.

3.4.3. Effect of recombinant bream insulin-like growth factor-I on HSP70 levels in whole blood

Like growth hormone, the physiological serum level of IGF-I of *Sparus sarba* has not been addressed in any study. Therefore, a wide range of doses (1ng/ml, 10ng/ml, 100ng/ml and 1000ng/ml) was used in the study. In one study on Atlantic salmon parr, the plasma IGF-I levels of the control and the stressed groups were shown to be about 100ng/ml and 130ng/ml respectively (McCormick *et al.*, 1998).

Since IGF-I is a factor in the somatotropic axis to mediate the growth-promoting effects of growth hormone and both growth hormone and IGF-I reduced the HSP70 levels in a dose-dependent manner, it is likely that growth hormone lowers HSP70 levels indirectly through stimulating blood cells to produce IGF-I which then decreases the HSP70 levels. Therefore, enhanced IGF-I status may be related to a reduced stress state. As with growth hormone, IGF-I also improves growth. The 2-year-old juvenile coho salmon received implants of osmotic minipumps containing recombinant bovine insulin-like growth factor-I (rbIGF-I) for 25 days was reported to have a doubled growth rate in length and an increased growth rate in weight by 40% (McCormick *et al.*, 1992). Thus IGF-I can be taken as an indicator of reduced stress. As yet no other works have addressed the direct effect of IGF-I on HSP70 levels in fish. One team found that the spinal cord trauma-induced HSP70 expression was significantly decreased in rats pretreated with IGF-I (Sharma *et al.*, 2000). Also, the role of IGF-I in the stress response in fish is still not established. In an experiment, Atlantic salmon parr were subjected to daily handling stress for 40 days. At 3 and 7 hours after the final handling, plasma GH and IGF- I levels were significantly higher

than the respective pre-stress controls (McCormick *et al.*, 1998). In mammals, acute thermal stress caused by elevated temperatures was reported to have no effect on plasma concentrations of IGF-I in lactating Holstein cows (McGuire *et al.*, 1991).

3.4.4. Effect of ovine prolactin on HSP70 levels in whole blood

As with growth hormone and IGF-I, the physiological serum level of prolactin of *Sparus sarba* has not been addressed in any study. Accordingly, a wide range of doses (1ng/ml, 10ng/ml, 100ng/ml and 1000ng/ml) was used in the study. In one study on rainbow trout, the prestress level of plasma prolactin in the control fish was shown to be between 2 and 4.5ng/ml. In the stressed fish, the plasma prolactin level was only slightly above 1ng/ml at 4 hours after the onset of confinement (Pottinger *et al.*, 1992). In another study on tilapia, the plasma levels of both ti-PRL_I and ti-PRL_{II} were shown to be about 3ng/ml. During confinement stress, the plasma ti-PRL_{II} level rose up to above 20ng/ml but the plasma ti-PRL_I level ranged between 10ng/ml and 20ng/ml (Auperin *et al.*, 1997)

The present study has shown that HSP70 levels in whole blood of *Sparus sarba* were reduced by ovine prolactin. Apart from the present study, daily intraperitoneal injection of ovine prolactin *in vivo* also reduced hepatic HSP70 levels in *Sparus sarba* significantly (Deane *et al.*, 1999a). In addition, the effect of two pharmacological drugs on hepatic HSP70 expression in silver sea bream was also investigated (Deane *et al.*, 2000a). These two drugs can regulate the endogenous prolactin synthesis. One was sulpiride which is a prolactin stimulant and another was bromocriptine which is a

prolactin suppressant. Daily intraperitoneal injections of sulpiride and ovine prolactin caused a decrease in hepatic HSP70 levels. On the contrary, administration of bromocriptine increased the levels of hepatic HSP70 significantly. Despite these studies, the role of prolactin in the stress response of fish still remains unclear. In one study on the effect of confinement stress, either confinement stress alone or a combination of 48 hour of confinement stress with concomitant deterioration in water quality was shown to decrease the plasma prolactin levels significantly in rainbow trout (Pottinger *et al.*, 1992). In contrast, acute handling stress had no effect on plasma prolactin concentration but chronic confinement stress increased the plasma concentration of prolactin in coho salmon (*Oncorhynchus kisutch*) (Avella *et al.*, 1991). On the other hand, prolactin was demonstrated to stimulate the immune response in fish by increasing macrophage phagocytosis and blood lymphocyte count. Exogenous administration of ovine prolactin in juvenile *Sparus sarba* enhanced macrophage phagocytic activity in terms of both percent phagocytosis and phagocytic index of macrophages of both pronephros and spleen (Narnaware *et al.*, 1998). Similar to growth hormone and IGF-I, ovine prolactin reduced the HSP70 levels in whole blood in a dose-dependent manner and therefore prolactin might also be related to a reduced stress state. However, based on the heterologous assay of the competition studies using iodinated ovine prolactin to bind to the tissue membranes of tilapia, both fish growth hormone and prolactin receptor sites recognize ovine prolactin due to structural similarity of molecules of growth hormone and prolactin from different species (Dauder *et al.*, 1990). The effect of homologous prolactin on blood HSP70 may not be the same as that of heterologous prolactin.

3.5. Conclusion

Cortisol did not affect the HSP70 levels in whole blood. There may not be a definite relationship between the classic stress hormone cortisol and the cellular stress response protein HSP70 expression in fish blood. Recombinant bream growth hormone, IGF-I and ovine prolactin all significantly reduced HSP70 levels in whole blood in a dose-dependent manner. The downregulation of HSP70 expression suggested that growth hormone, IGF-I and prolactin might be related to a reduced stress state. These three hormones may have a protective effect on stress tolerance.

Chapter 4:

Effect on HSP70 expression in whole blood of *Sparus sarba* acclimated to various salinities

Chapter 4: Effect on HSP70 expression in whole blood of silver sea bream acclimated to various salinities

4.1. Introduction

Euryhaline teleosts display a myriad of physiological responses to salinity and most responses are associated with osmoregulation. For example, plasma osmolarity and concentrations of Na^+ and Cl^- elevate temporarily after the transfer from fresh water to seawater. For example, plasma concentrations of Na^+ and Cl^- elevated in Mozambique tilapia (*Oreochromis mossambicus*) 1 day after transfer from fresh water to seawater (Morgan *et al.*, 1997). Besides, the gill Na^+-K^+-ATPase activity and the number of branchial chloride cells usually increase in seawater. In a study on the euryhaline killifish *Fundulus heteroclitus* and parr and presmolt of Atlantic salmon (*Salmo salar*), gill Na^+-K^+-ATPase activity of both fish was shown to increase following the transfer from low salinity of 0.1 ppt to high salinity of 25-35 ppt (Mancera and McCormick, 2000). Gill chloride cells were reported to increase in size in Atlantic salmon exposed to seawater and decrease in size in fish held in fresh water (Birt and Green, 1993). Likewise, alternation of branchial chloride cell morphology is also critical for euryhalinity. For instance, the euryhaline killifish, the tight junction between adjacent chloride cells becomes shallow following seawater adaptation and assumes importance for it in secreting NaCl. On the contrary, in fresh water, the tight junction between adjacent chloride cells is mostly deep as the branchial ionic permeability of freshwater-adapted fish is low (Karnaky, 1998). Generally speaking, plasma levels of cortisol and growth hormone would elevate during transfer from fresh water to seawater. Higher cortisol release and a marked hyperplasia of the

interrenal cells were observed in the seawater-adapted tilapia (Balm *et al.*, 1995). In contrast, plasma level of growth hormone was found to decrease significantly in the teleost tilapia after the transfer from seawater to fresh water (Yada *et al.*, 1994).

There are only a few works that have studied the effect of salinity stress on expression of heat shock proteins in intact fish. In one study, the effects of long-term (8 months) salinity adaptation on black sea bream (*Mylio macrocephalus*) on expression of various heat shock proteins were examined. Hepatic HSP90, HSP70 and HSP60 levels were shown to be lowest in fish reared in an iso-osmotic salinity (12 ppt) and highest at salinity extremes of 6 ppt and 50 ppt (Deane *et al.*, 2001a). The purpose of this part of study was to examine the effect on HSP70 expression in whole blood of euryhaline silver sea bream acclimated to various salinities, including a strongly hyper-osmotic salinity (50 ppt), the salinity of normal seawater (33 ppt), the iso-osmotic salinity of sea bream (12 ppt), a hypo-osmotic salinity (6 ppt) and the salinity of fresh water (0 ppt). Iso-osmotic adaptation is believed to be beneficial to aquaculture as it has been reported to enhance growth by minimizing the metabolic cost of osmoregulation and stimulate the non-specific immune response of fish (Narnaware *et al.*, 2000). As HSP70 expression can serve as a reliable bioindicator of stress, the stress levels of fish in different salinities were determined by this *in vivo* experiment.

4.2. Materials and methods

4.2.1. Overall experimental design

Silver sea breams (*Sparus sarba*) were acclimated to various salinities (0, 6, 12, 33 and 50 ppt) respectively for one month (n=7 per group). Fish were then sacrificed and blood was taken from the caudal vessels by heparinized syringes. The heparinized blood was centrifuged at 10,000 rpm for 10 minutes. The supernatants were discarded and the pellets were washed in 1ml DMEM (GibcoBRL). The suspensions were centrifuged at 10,000 rpm for 10 minutes and pelleted. The blood pellets were immediately frozen in liquid nitrogen and stored in deep freezer at -70°C for later analysis. All the soluble proteins of the blood were extracted and the blood HSP70 expression was assessed by indirect ELISA.

4.2.2. Protein extraction and quantification, indirect ELISA, gel electrophoresis, and immunoblotting (Western blotting)

Procedures of protein extraction and quantification, indirect ELISA, gel electrophoresis and immunoblotting were exactly the same as those described in Materials and Methods in Chapter 3.

4.2.3. Statistical analysis

All the data in the study are presented as means ± standard error (SEM) and were subjected to a one-way ANOVA to test for significance. Subsequent significance was delineated by a Student-Newman-Kuels test (SigmaStat statistical software, Jandel Scientific).

4.3. Results

Silver sea bream adapted to an iso-osmotic salinity (12 ppt) had a significantly lower level of HSP70 in whole blood than those reared in all different salinities (Figs. 4.1. and 4.2.). The mean HSP70 level of whole blood in *Sparus sarba* reared in 12 ppt was only 3.93 ± 0.88 ng/μg total soluble protein. On the contrary, the HSP70 expression of whole blood in fish held in a hypo-osmotic salinity of 6 ppt was highest. The mean blood HSP70 of sea breams kept in 6 ppt was 29.87 ± 1.53 ng/μg total soluble protein. The HSP70 levels in fish held in 50 ppt were the second highest. The mean blood HSP70 level of silver sea breams acclimated in 50 ppt was 21.8 ± 1.12 ng/μg total soluble protein. When compared to the HSP70 levels of fish reared in seawater (10.62 ± 1.06 ng/μg total soluble protein), the HSP70 expression in fish kept in fresh water (15.14 ± 0.54 ng/μg total soluble protein) was significantly higher.

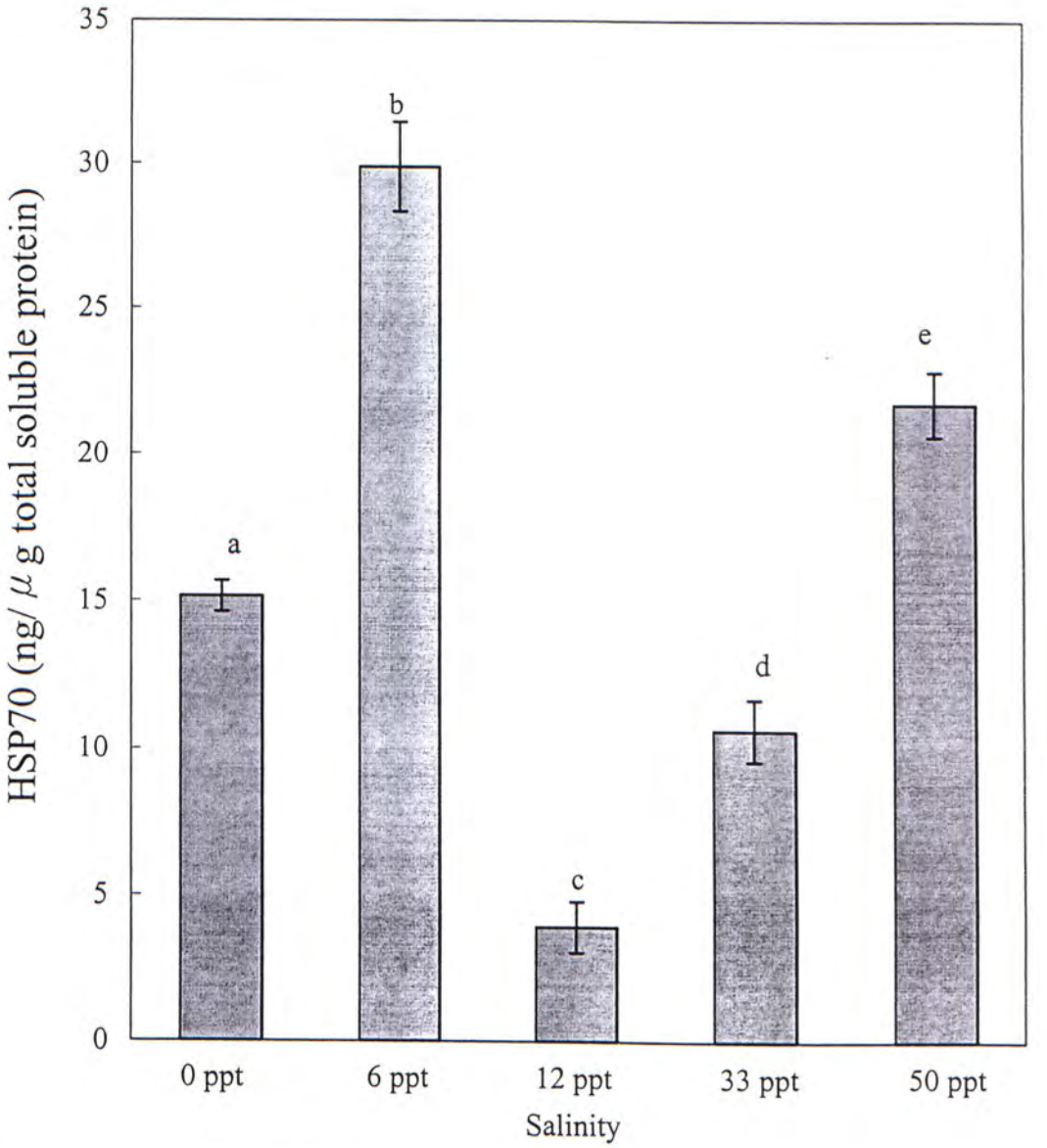

Figure 4.1. HSP70 expression in whole blood of *Sparus sarba* acclimated at 0 ppt, 6 ppt, 12 ppt, 33 ppt and 50 ppt. Values with different alphabets are significantly different from each other ($P<0.05$), Student-Newman-Kuels Multiple Comparison.

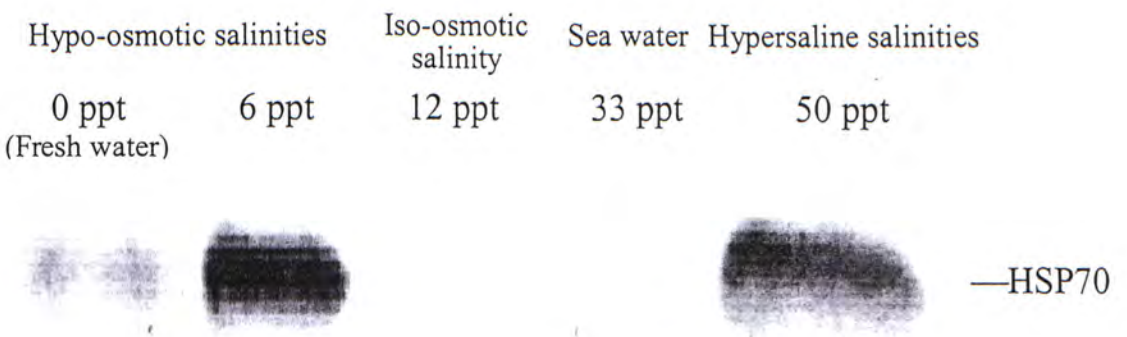

Figure 4.2. Representative western blot analysis of HSP70 in whole blood of *Sparus sarba* acclimated at 0 ppt, 6 ppt, 12 ppt, 33 ppt and 50 ppt.

4.4. Discussion

The results showed that the HSP70 levels of whole blood in fish held in an iso-osmotic salinity of 12 ppt was lowest. This implied that iso-osmotic salinity would bring about the least stress level. Similar pattern was observed in a study on the effect of long-term salinity adaptation on HSP70 levels in liver of black sea bream (*Mylio macrocephalus*) (Deane *et al.*, 2001a). The hepatic HSP70 levels were lowest in a 12 ppt environment and highest at salinity extremes of 6 ppt and 50 ppt. *In vitro* exposure to salinity stress also induces the HSP70 synthesis in other tissues of fish. HSP70 in isolated branchial lamellae of anadromous Atlantic salmon (*Salmo salar*) was also induced in response to *in vitro* exposure to hyperosmotic conditions created by NaCl for 12 hours (Smith *et al.*, 1999).

Some papers have reported that iso-osmotic salinity would bring about the least stress level. As stressed fish commonly show reduced growth, growth rate is regarded as a reliable stress index in fish (Wendelaar Bonga, 1997). The growth rates of sea bass (*Lates calcarifer*) (Woo and Chiu, 1994), *Sparus sarba* (Woo and Kelly, 1995) and tilapia (*Oreochromis niloticus*) (Woo *et al.*, 1997) held in an iso-osmotic salinity of 15 ppt were significantly greater than fish at other salinities. On the contrary, oxygen consumption was lowest in *Sparus sarba* at 15 ppt. The metabolic cost of osmoregulation in iso-osmotic water is lower than in either hyper-osmotic or hypo-osmotic water. Energy can be saved owing to the lowest costs of ion and water regulation in iso-osmotic water (Febry and Lutz, 1987). In an iso-osmotic environment, the ionic gradients between blood and ambient water were minimal. Since the passive influx of ions from the iso-osmotic medium is reduced to a minimum,

the energetic costs for active ion transport at gills and intestine of seawater-acclimated fish to iso-omostic water can be consequently reduced. On the other hand, the passive influx of water from the iso-osmotic medium is reduced to a minimum, the compensatory volume of urine produced in freshwater-acclimated fish can be therefore decreased to a minimum level. Gill Na^+-K^+-ATPase activity was lowest in *Oncorhynchus kisutch* coho salmon smolts (Morgan and Iwama, 1998) and Mozambique tilapia (*Oreochromis mossambicus*) (Kültz *et al.*, 1992) acclimated to iso-osmotic salinity compared to fresh water and sea water.

Iso-osmotic adaptation has also been reported to stimulate the immune response of teleosts. The levels of macrophage phagocytic activity in terms of percent phagocytosis of macrophages of both pronephros and spleen in juvenile black sea bream adapted to iso-osmotic medium of 12 ppt and fed with a only 5% ration size were similar to fish fed with a 10% ration size (Narnaware *et al.*, 2000). Thus it appears that the energy saved from reduced osmotic output in an iso-somotic medium can be channeled to benefit other biological functions including growth and immune function.

In addition, the least stress level in iso-osmotic salinity can also be inferred from the data on liver IGF-I expression. Based on the study on the effect of long-term salinity adaptation on liver IGF-I mRNA expression in black sea bream by Deane *et al.* (2001a), IGF-I expression was lowered in the hyposaline salinity (6 ppt) and the hypersaline salinity (50 ppt). However, the HSP70 levels were highest at these extremes of salinity. In contrast, at the iso-osmotic salinity of 12 ppt, the IGF-I levels were highest. This was correlated with the lowest HSP70 expression. Lowest

HSP70 expression can be associated with an environment with least stress level (Deane *et al.*, 2001a). Classic stress hormone, cortisol, is a primary stress response parameter. Serum levels of cortisol were reported to be lower in emperor angel fish (*Pomacanthus imperator*) acclimated to iso-osmotic salinity of 15 ppt (Woo and Chung, 1995).

Furthermore, the present study showed that freshwater acclimation resulted in an even lower level of blood HSP70 than that in 6 ppt. However, judging from the osmotic gradient between the environment and the body fluids, 0 ppt should be a much more stressful environment. By using HSP70 expression as a bioindicator of stress in fish, the stress levels of fish adapted to fresh water were lower than those in 6 ppt and 50 ppt environments. The reason why this is so is not known. However, there are reports that stress level can be influenced by salinity. Chronic stress such as confinement was reported to be more stressful to the same species of fish in salt water than to those in fresh water. For instance, in one study on coho salmon (*Oncorhynchus kisutch*), the smolts that had been fully adapted to fresh water or seawater were subsequently subjected to confinement (Avella *et al.*, 1991). By using plasma cortisol as an indicator of stress, the plasma cortisol levels of the stressed smolts in seawater were higher and remained elevated by 5 days after onset of stress. Also, plasma Na^+ was significantly higher in the seawater stressed smolts at 5 days after the stress had started. Likewise, seawater smolts in the stressed group had some mortality (Avella *et al.*, 1991).

4.5. Conclusion

The lowest HSP70 levels in whole blood were found in fish adapted to an iso-osmotic salinity of 12 ppt. HSP70 levels were markedly elevated in 50 ppt and 6 ppt environments. Freshwater acclimation resulted in return to lower levels. The results indicated that iso-osmotic salinity would bring about the least stress level. This supported that iso-osmotic environment was an environment of minimal stress which is conducive for growth stimulation. 50 ppt and 6 ppt were the most stressful salinities to *Sparus sarba*. Compared to 50 ppt and 6 ppt, the stress levels of fish in fresh water were lower.

Chapter 5:

General discussion and conclusion

Chapter 5: General discussion and conclusion

In this thesis, the HSP70 expression of *Sparus sarba* was addressed and whole blood was used as an *in vitro* model for study. In the first and second parts, the HSP70 expression in whole blood exposed to *in vitro* thermal stress and hormone treatments was examined. In the third part, fish were subjected to *in vivo* osmotic stress and the blood HSP70 expression was then investigated. In this study, the HSP70 expression was assessed by indirect ELISA. The indirect ELISA for sea bream whole blood HSP70 was developed, as the standard curve constructed using bovine HSP70 and the titration curve of serially diluted whole blood protein displayed parallelism. Since HSP70 synthesis can be induced by many proteotoxic stresses and these proteins are highly conserved, HSP70 can serve as a useful molecular biomarker of environmental stress (Ryan and Hightower, 1996). Therefore, the stress levels in fish can be determined by using HSP70 expression as a bioindicator of stress.

In the first part of study, upregulation of blood HSP70 levels of *Sparus sarba* was observed following 2-hour *in vitro* exposure to thermal stress including cold shock (12°C), heat shock (27°C) and severe heat shock (32°C). The temperature at which HSP70 synthesis was induced was between 19 and 27°C and maximal HSP70 induction occurred at 32°C. In one study, HSP70 synthesis in red blood cells of eurythermal killifish *Fundulus heteroclitus* was induced by heat shock at 32.5°C and the HSP70 mRNA levels has been shown to increase further after heat shock at 40°C (Koban *et al.*, 1991). In this respect, the HSP70 induction profile of whole blood in *Sparus sarba* is similar to that of red blood cells in *Fundulus heteroclitus*. The HSP70 levels in whole blood also significantly increased at 12°C from a control

temperature of 19°C. However, there are not many works which addressed the induction of HSP70 synthesis in fish after cold shock. *In vivo* cold shock at 16°C was found to have no effect on hepatic HSP70 expression in *Sparus sarba* (Deane *et al.*, 1999a, 2000a). The results of this study support the notion that both heat and cold stress are potent stimuli for blood HSP70 upregulation in fish.

Since the endocrine regulation of HSP70 expression in fish is not clear, one of the aims of the present study was to establish whether key hormones directly regulate blood HSP70 expression in *Sparus sarba*. *In vitro* exposure to cortisol, the classic stress hormone, was found to have no effect on the HSP70 levels in whole blood. In another study on *Sparus sarba*, daily administration of exogenous cortisol was also failed to alter the hepatic HSP70 expression (Deane *et al.*, 1999). These two studies support the contention that cortisol might not bear a definite relationship to blood HSP70 expression in fish. Recombinant bream growth hormone (rbGH), insulin-like growth factor- I (rbIGF-I) and ovine prolactin (oPRL) all significantly reduced HSP70 levels in whole blood in a dose-dependent manner. Similar results were demonstrated in a study on *Sparus sarba*. Daily intraperitoneal injections of rbGH and oPRL were found to cause a significant decrease in hepatic HSP70 levels (Deane *et al.*, 1999). Since HSP70 expression indicates the stress levels, the results of these two studies supports the notion that growth hormone, IGF-I and prolactin might be related to a reduced stress state and have a protective effect on stress tolerance. However, in both studies, heterologous prolactin was used instead of the homologous one. Based on the heterologous assay of the competition studies using iodinated ovine prolactin to bind to the tissue membranes of tilapia, both fish growth hormone and prolactin receptor sites recognize ovine prolactin (Dauder *et al.*, 1990). The possibility that

homologous prolactin does not have the same effect on blood HSP70 expression cannot be eliminated.

Lastly, the effect of a 4-week salinity adaptation on HSP70 expression in whole blood of euryhaline teleost *Sparus sarba* was examined in this study. This is an *in vivo* experiment. The lowest blood HSP70 expression was found in fish adapted to an iso-osmotic salinity of 12 ppt. The HSP70 levels were highest in 50 ppt and 6 ppt environments. Similar pattern was observed in a study on the effect of long-term salinity adaptation on HSP70 levels in liver of black sea bream (*Mylio macrocephalus*) (Deane and Woo, unpublished). The hepatic HSP70 expression was lowest in a 12ppt environment and highest at salinity extremes of 6 ppt and 50 ppt. Iso-osmotic adaptation has been reported to enhance fish growth and stimulate the non-specific immune response of fish (Narnaware *et al.*, 2000). By using HSP70 expression as a bioindicator of stress, the results indicated that iso-osmotic salinity would bring about the least stress level while 50 ppt and 6 ppt were the most stressful salinities to *Sparus sarba*. This study further confirms the hypothesis that iso-osmotic environment is an environment of minimal stress.

Reference

Airaksinen, S., Rabergh, C. M. I., Sistonen, L. and Nikinmaa, M. (1998) Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. *Journal of Experimental Biology*, 201: 2543-2551.

Allen, S. P., Polazzi, J. O. Gierse, J. K. and Easton, A. M. (1992) Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in *Escherichia coli*. *Journal of Bacteriology*, 6938-6947.

Arrigo, A. P. and Landry, J. (1994) Expression and function of low-molecular weight heat shock proteins. In: *The Biology of Heat Shock Proteins and Molecular Chaperones*, pp. 335-373. Edited by R. I. Morimoto, A. Tissières and C. Georgopoulos. Cold Spring Harbor Laboratory Press, New York.

Auperin, B., Baroiller, J. F., Ricordel, M. J., Fostier, A. and Prunet, P. (1997) Effect of confinement stress on circulating levels of growth hormone and two prolactins in freshwater-adapted tilapia (*Oreochromis niloticus*). *General and Comparative Endocrinology*, 108: 35-44.

Avella, M., Schreck, C. B. and Prunet, P. (1991) Plasma prolactin and cortisol concentrations of stressed coho salmon, *Oncorhynchus kisutch*, in fresh water or salt water. *General and Comparative Endocrinology*, 81: 21-27.

Ball, J. N. (1969) Prolactin (fish prolactin or paralactin) and growth hormone. In: *Fish*

Physiology, Vol. II, pp. 207-240. Edited by W. S. Hoar and D. J. Randall. Academic Press, Inc., New York.

Balm, P. H. M., Haenen, H. E. M. G. and Wendelaar-Bonga, S. E. (1995) Regulation of interrenal function in freshwater and sea water adapted tilapia (*Oreochromis mossambicus*). *Fish Physiology and Biochemistry*, 14: 37-47.

Batten, T. F. C. and Ingleton, P. M. (1987) The hypothalamus and pituitary gland. In: *Fundamentals of Comparative Vertebrate Endocrinology*, pp. 281-409. Edited by I. Chester-Jones, P. M. Ingleton and J. G. Philips. Plenum Press, New York.

Benndorf, R., Hayess, K., Ryazantsev, S., Wieske, M., Behlke, J. and Lutsch, G. (1994) Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. *Journal of Biological Chemistry*, 269: 20780-20784.

Benali, N., Ferjoux, G., Puente, E., Buscail, L. and Susini, C. (2000) Somatostatin receptors. *Digestion*, 62: 27-32.

Bentley, P. J. (1998a) Hormones and nutrition. In: *Comparative Vertebrate Endocrinology*, pp. 223-268. Edited by P. J. Bentley. Cambridge University Press, United Kingdom.

Bentley, P. J. (1998b) The chemical structure, polymorphism, evolution of hormones. In: *Comparative Vertebrate Endocrinology*, pp. 65-176. Edited by P. J. Bentley.

Cambridge University Press, United Kingdom.

Birt, T. P. and Green, J. M. (1993) Acclimation to seawater of dwarf nonanadromous Atlantic salmon, *Salmo salar*. *Canadian Journal of Zoology*, 71: 1912-1916.

Blake, M. J., Udelsman, R., Feulner, G. J., Norton, D. D. and Holbrook, N. J. (1991) Stress-induced heat shock protein 70 expression in adrenal cortex: An adrenocorticotropic hormone-sensitive, age-dependent response. *Proceedings of the National Academy of Sciences of the United States of America*, 88: 9873-9877.

Bochkareva, E. S., Lissin, N. M., Flynn, G. C., Rothman, J. E. and Girshovich, A. S. (1992) Positive cooperativity in the functioning of molecular chaperone GroEL. *Journal of Biological Chemistry*, 267: 6796-6800.

Brodsky, J. L., Goeckeler, J. and Schekman, R. (1995) BiP and Sec63p are required for both co- and posttranslational protein translocation into the yeast endoplasmic reticulum. *Proceedings of the National Academy of Sciences of the United States of America*, 92: 9643-9646.

Bjornsson, B. T. (1997) The biology of salmon growth hormone: from daylight to dominance. *Fish Physiology and Biochemistry*, 17: 9-24.

Calduch-Giner, J. A., Sitja, B. A., Alvarez, P. P. and Perez, S. J. (1997) Growth hormone as an in vitro phagocyte-activating factor in the gilthead sea bream (*Sparus aurata*). *Cell and Tissue Research*, 287: 535-540.

Carragher, J. F. and Rees, C. M. (1994) Primary and secondary responses in golden perch, Macquaria ambigua. *Comparative Biochemistry and Physiology*, 107A: 49-56.

Carter-Su, C., Schwartz, J. and Smith, L. S. (1996) Molecular mechanism of growth hormone action. *Annual Review of Physiology*, 58: 187-207.

Cavari, B., Noso, T. and Kawauchi, H. (1995) Somatolactin, a novel pituitary protein: Isolation and characterization from *Sparus aurata*. *Molecular Marine Biology and Biotechnology*, 4: 117-122.

Chan, D. K. O. and Woo, N. Y. S. (1978) Effect of cortisol on the metabolism of the eel, *Anguilla japonica*. *General and Comparative Endocrinology*, 35: 205-215.

Chiang, H. L., S. R. Terlecky, C. P. Plant and J. F. Dice (1989) A role for a 70-kilodalton heat shock protein in lysosomal proteolysis of intracellular proteins. *Science*, 246: 282-285.

Coux, O., Tanaka, K. and Goldberg, A. L. (1996) Structure and functions of the 20S and 26S proteasomes. *Annual Review of Biochemistry*, 65: 801-847.

Currie, S., Moyes, C. D. and Tufts, B. L. (2000) The effects of heat shock and acclimation temperature on hsp70 and hsp30 mRNA expression in rainbow trout: In vivo and in vitro comparisons. *Journal of Fish Biology*, 56: 398-408.

Currie, S. and Tufts, B. L. (1997) Synthesis of stress protein 70 (Hsp70) in rainbow

trout (*Oncorhynchus mykiss*) red blood cells. *Journal of Experimental Biology*, 200: 607-614.

Dauder, S., Young, G., Hass, L. and Bern, H. A. (1990) Prolactin receptors in liver, kidney, and gill of the tilapia (*Oreochromis mossambicus*): Characterization and effect of salinity on specific binding of iodinated ovine prolactin. *General and Comparative Endocrinology*, 77: 368-377.

Deane, E. E., Kelly, S. P., Lo, C. K. M. and Woo, N. Y. S. (1999a) Effects of GH, prolactin and cortisol on hepatic heat shock protein 70 expression in a marine teleost *Sparus sarba*. *Journal of Endocrinology*, 161: 413-421.

Deane, E. E., Kelly, S. P. and Woo, N. Y. S. (1999b) Hormonal modulation of branchial Na+-K+-ATPase subunit mRNA in a marine teleost *Sparus sarba*. *Life Sciences*, 64: 1819-1829.

Deane, E. E., Kelly, S. P., Chow, I. N. K. and Woo, N. Y. S. (2000a) Effect of a prolactin pharmacological stimulant (sulpiride) and suppressant (bromocriptine) on heat shock protein 70 expression in silver sea bream, *Sparus sarba*. *Fish Physiology and Biochemistry*, 22: 125-133.

Deane, E. E., Kelly, S. P. and Woo, N. Y. S. (2000b) Hypercortisolemia does not affect the branchial osmoregulatory responses of the marine teleost *Sparus sarba*. *Life Sciences*, 66: 1435-1444.

Deane, E. E., Kelly, S. P., Luk, J. C. Y. and Woo, N. Y. S. (2001a) Chronic salinity adaptation modulates hepatic HSP and IGF-I expression in black sea bream. (Submitted to Marine Biotechnology)

Deane, E. E., Li, J. and Woo, N. Y. S. (2001b) Hormonal status and phagocytic activity in sea bream infected with vibriosis. *Comparative Biochemistry and Physiology*, 129B: 687-693.

de Vos, A. M., Ultsch, M. and Kossiakoff, A. A. (1992) Human growth hormone and extracellular domains of its receptor: crystal structure of the complex. *Science*, 255: 306-312.

Dice, J. F., Agarraberes, F., Kirven-Brooks, M., Terlecky, L. J. and Terlecky, S. R. (1994) Heat shock 70-kDa proteins and lysosomal proteolysis. In: *The Biology of Heat Shock Proteins and Molecular Chaperones*, pp. 137-151. Edited by R. I. Morimoto, A. Tissières and C. Georgopoulus. Cold Spring Harbor Laboratory Press, NewYork.

Dietz, T. J. and Somero, G. N. (1993) Species and tissue-specific synthesis patterns for heat-shock proteins HSP70 and HSP90 in several marine teleost fishes. *Physiological Zoology*, 66: 863-880.

Duan, C., Ding, J., Li, Q., Tsai, W. and Pozios, K. (1999) Insulin-like growth factor binding protein 2 is a growth inhibitory protein conserved in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, 96: 15274-15279.

Duan, C., Plisetskaya, E. M. and Dickhoff, W. W. (1995) Expression of insulin-like growth factor I in normally and abnormally developing coho salmon (*Oncorhynchus kisutch*). *Endocrinology*, 136: 446-452.

Ehrnsperger, M., Buchner, J. and Gaestel, M. (1998) Structure and function of small heat-shock proteins. In: *Molecular Chaperones in the Life Cycle of Proteins*, pp. 533-575. Edited by A. L. Fink and Y. Goto. Marcel Dekker, Inc., New York.

Ferby, R. and Lutz, P. (1987) Energy partitioning in fish: the activity-related cost of osmoregulation in euryhaline cichlid. *Journal of Experimental Biology*, 128:63-85.

Finley, D., Özkaynak, E. and Varshavsky, A. (1987) The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. *Cell*, 48: 1035-1046.

Flik, G., Atsma, W., Fenwick, J. C., Rentier-Delrue, F. Smal, J. and Wendelaar Bonga, S. E. (1993) Homologous recombinant growth hormone and calcium metabolism in the tilapia, *Oreochromis mossambicus*, adapted to fresh water. *Journal of Experimental Biology*, 185: 107-119.

Flik, G., Fenwick, J. C., Kolar, Z., Mayer-Gostan, N. and Wendelaar Bonga, S. E. (1986) Effects of ovine prolactin on calcium uptake and distribution in *Oreochromis mossambicus*. *American Journal of Physiology*, 250: R161-R166.

Flik, G., Rentier-Delrue, F. and Wendelaar-Bonga, S. E. (1994) Calcitropic effects of

recombinant prolactins in *Oreochromis mossambicus*. *American Journal of Physiology*, 266: R1302-R1308.

Flik, G., van der Velden, J. A., Dechering, K. J., Verbost, P. M., Schoenmakers, T. J. M., Kolar, Z. I. and Wendelaar Bonga, S. E. (1993) Ca^{2+} and Mg^{2+} transport in gut and gills of tilapia, Oreochromis mossambicus: a review. *Journal of Experimental Biology*, 265: 356-365.

Forsyth, R. B., Candido, E. P. M., Babich, S. L. and Iwama, G. K. (1997) Stress protein expression in coho salmon with bacterial kidney disease. *Journal of Aquatic Animal Health*, 9: 18-25.

Foskett, J. K. and Scheffey, C. (1982) The chloride cell: definitive identification as the salt-secretory cell in teleosts. *Science*, 215: 164-166.

Funkenstein, B., Silbergeld, A., Cavari, B. and Laro, Z. (1989) Growth hormone increases plasma levels of insulin-like growth factor (IGF-I) in a teleost, the gilthead seabream (*Sparus sarba*). *Journal of Endocrinology*, 120: R19-R21.

Gething, M. J. and Sambrook, J. (1992) Protein folding in the cell. *Nature*, 355: 33-45.

Gottesman, S., Clark, W. P. and Maurizi, M. R. (1990) The ATP-dependent Clp protease of *Escherichia coli*: Sequence of clpA and identification of a Clp-specific substrate. *Journal of Biological Chemistry*, 265: 7886-7893.

Haas, I. G. and Wabl, M. (1983) Immunoglobulin heavy chain binding protein. *Nature*, 306: 387-389.

Hazon, N. and Balment, R. J. (1998) Endocrinology. In: *The Physiology of Fishes*, pp. 441-463. Edited by D. H. Evans. CRC Press LLC, Boca Raton, Florida.

Heim, M. H. (1999) The JAK-STAT pathway: Cytokine signalling from the receptor to the nucleus. *Journal of Receptor and Signal Transduction Research*, 19: 75-120.

Herndon, T. M., McCormick, S. D. and Bern, H. A. (1991) Effects of prolactin on chloride cells in opercular membrane of seawater-adapted tilapia. *General and Comparative Endocrinology*, 83: 283-289.

Hooghe-Peters, E. L. and Hooghe, R. (1995) Cytokine-hemoprotein receptors. In: *Growth Hormone, Prolactin and IGF-I as Lymphohemopoietic Cytokines*, pp. 115-136. Edited by E. L. Hooghe-Peters and R. Hooghe. Springer-Verlag, Austin, Taxas.

Hoskins, J. R., Singh, S. K., Maurizi, M. R. and Wickner, S. (2000) Protein binding and unfolding by the chaperone ClpA and degradation by the protease ClpAP. *Proceedings of the National Academy of Sciences of the United States of America*, 97: 8892-8897.

Hwang, B., Woo, K., Goldberg, A. and Chung, C. (1988) Protease Ti, a new ATP-dependent protease in *Escherichia coli*, contains protein-activated ATPase and proteolytic functions in distinct subunits. *Journal of Biological Chemistry*, 263:

8727-8734.

Iwama, G. K., Thomas, P. T., Forsyth, R. B. and Vijayan, M. M. (1998) Heat shock protein expression in fish. *Reviews in Fish Biology and Fisheries*, 8: 35-56.

Jakob, U., Gaestel, M., Engels, K. and Buchner, J. (1993) Small heat shock proteins are molecular chaperones. *Journal of Biological Chemistry*, 268: 1517-1520.

Joplin, K. H., Yocum, G. D. and Denlinger, D. L. (1990) Cold shock elicits expression of heat shock proteins in the flesh fly, *Sarcophaga crassipalpis*. *Journal of Insect Physiology*, 36: 825-834.

Kabakov, A. E. and Gabai, V. L. (1997) Heat shock proteins and the regulation of heat shock gene expression in eukaryotes. In: *Heat Shock Proteins and Cytoprotection: ATP-deprived Mammalian Cells*, pp. 1-19. Edited by A. E. Kabakov and V. L. Gabai. Springer-Verlag, Austin, Taxas.

Kakizawa, S., Kaneko, T., Hasegawa, S. and Hirano, T. (1993) Activation of somatolactin cells in the pituitary of the rainbow trout *Oncorhynchus mykiss* by low environmental calcium. *General and Comparative Endocrinology*, 91: 298-306.

Kakizawa, S., Kaneko, T., Hasegawa, S. and Hirano, T. (1995) Effects of feeding, fasting, background adaptation, acute stress, and exhaustive exercise on the plasma somatolactin concentrations in rainbow trout. *General and Comparative Endocrinology*, 98: 137-146.

Kakizawa, S., Kaneko, T. and Hirano, T. (1996) Elevation of plasma somatolactin concentrations during acidosis in rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology*, 199(5): 1043-1051.

Karnaky, K. J. Jr. (1998) Osmotic and ionic regulation. In: *The Physiology of Fishes*, pp. 157-176. Edited by D. H. Evans. CRC Press LLC, Boca Raton, Florida.

Kelly, P. A., Finidori, M. C., Postel-Vinay, M., Edery, M., Goujon, L., Sotiropoulos, H., Dinerstein, H. and Lebrun, J. J. (1995) GH binding protein, GH receptors, and domains involved in signal transduction. In: *GHRH, GH, and IGF-I: Basic and Clinical Advances*, pp. 25-37. Edited by M. R. Blackman, S.M. Harman, J., Roth and J. R. Shapiro. Springer-Verlag, New York.

Kelly, S. P. and Woo, N. Y. S. (1999) Cellular and biochemical characterization of hyposmotic adaptation in a marine teleost, *Sparus sarba*. *Zoological Science Tokyo*, 16: 505-514.

Koban, M., Yup, A. A., Agellon, L. B. and Powers, D. A. (1991) Molecular adaptation to environmental temperature: Heat-shock response of the eurythermal teleost *Fundulus heteroclitus*. *Molecular Marine Biology and Biotechnology*, 1: 1-17.

Kültz, D., Bastrop, R., Jürss, K. and Siebers, D. (1992) Mitochondria-rich (MR) cells and the activities of the sodium, potassium ATPase and carbonic anhydrase in the gill and opercular epithelium of *Oreochromis mossambicus* adapted to various salinities. *Comparative Biochemistry and Physiology*, 102B: 293-301.

Laurent, P. and Perry, S. F. (1990) Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. *Cell and Tissue Research*, 259: 429-442.

Laios, E., Rebeyka, I. M. and Prody, C. A. (1997) Characterization of cold-induced heat shock protein expression in neonatal rat cardiomyocytes. *Molecular and Cellular Biochemistry*, 173: 153-159.

Lam, T. J. (1972) Prolactin and hydromineral metabolism in fishes. *General and Comparative Endocrinology*, Supplement 3: 328-338.

Lavoie, J. N., Lambert, H., Hickey, E., Weber, L. A. and Landry, J. (1995) Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. *Molecular and Cellular Biology*, 15: 505-516.

Lee, A. C. (1992) Mammalian stress response: Induction of the glucose-regulated protein family. *Current Opinion in Cell Biology*, 4: 267-273.

Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. and Zylicz, M. (1991) *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proceedings of the National Academy of Sciences of the United States of America*, 88: 2874-2878.

Ludwig, S., Engel, K., Hoffmeyer, A., Sithanandam, G., Neufeld, B., Palm, D., Gaestel,

M. and Rapp, U. R. (1996) 3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways. *Molecular and Cellular Biology*, 16: 6687-6697.

Lyons, A. M., Mooney, R., Lying, F., Cottell, D. and Mothersill, C. (1998) The effects of cadmium exposure on the cytology and function of primary cultures from rainbow trout. *Cell Biochemistry and Function*, 16: 1-13.

Mancera, J. M. and McCormick, S. D. (2000) Rapid activation of gill Na^+-K^+-ATPase in the euryhaline teleost *Fundulus heteroclitus*. *Journal of Experimental Zoology*, 287: 263-274.

McCormick, S. D. (1990) Cortisol directly stimulates differentiation of chloride cells in tilapia opercular membrane. *American Journal of Physiology*, 259: R857-R864.

McCormick, S. D. (1996) Effects of growth hormone and insulin-like growth factor I on salinity tolerance and gill Na^+-K^+-ATPase in Atlantic salmon (*Salmo salar*): interaction with cortisol. *General and Comparative Endocrinology*, 101: 3-11.

McCormick, S. D., Kelley, K. M., Young, G., Nishioka, R. S. and Bern, H. A. (1992) Stimulation of coho salmon growth by insulin-like growth factor I. *General and Comparative Endocrinology*, 86: 398-406.

McCormick, S. D., Sakamoto, T., Hasegawa, S. and Hirano, T. (1991) Osmoregulatory action of insulin-like growth factor in rainbow trout (*Oncorhynchus kisutch*). *Journal*

of Endocrinology, 130: 87-92.

McCormick, S. D., Shrimpton, J. M., Carey, J. B., O'-Dea, M. F., Sloan, K. E., Moriyama, S. and Bjornsson, B. T. (1998) Repeated acute stress reduces growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol. *Aquaculture*, 168: 221-235.

McGuire, M. A., Beede, D. K., Collier, R. J., Buonomo, F. C., Delorenzo, M. A., Wilcox, C. J., Huntington, G. B. and Reynolds, C. K. (1991) Effects of acute thermal stress and amount of feed intake on concentrations of somatotropin, insulin-like growth factor (IGF)-I and IGF-II, and thyroid hormones in plasma of lactating Holstein cows. *Journal of Animal Science*, 69: 2050-2056.

McLean, E., Donaldson, E. M., Dye, H. M. and Souza, L. M. (1990) Growth acceleration of coho salmon (*Oncorhynchus kisutch*) following oral administration of recombinant bovine somatotropin. *Aquaculture*, 91: 197-203.

McLean, E., Teskeredzic, E., Donaldson, E. M., Teskeredzic, Z., Cha, Y., Sittner, R. and Pitt, C. G. (1992) Accelerated growth of coho salmon *Oncorhynchus kisutch* following sustained release of recombinant porcine somatotropin. *Aquaculture*, 103: 377-387.

Mehlen, P., Hickey, E., Weber, L. A. and Arrigo, A. P. (1997) Large unphosphorylated aggregates as the active form of hsp28 which controls intracellular reactive oxygen species and glutathione levels and generates a protection against TNFalpha in

NIH-3T3-ras cells. *Biochemical and Biophysical Research Communications*, 241: 187-192.

Melnick, J., Aviel, S. and Argon, Y. (1992) The endoplasmic reticulum stress protein GRP94, in addition to BiP, associates with unassembled immunoglobulin chains. *Journal of Biological Chemistry*, 267: 21303-21306.

Melnick, J., Dul, J. R. and Argon, Y. (1994) Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. *Nature*, 370: 373-375.

Miron, T., Vancompernolle, K., Vandekerckhove, J., Wilchek, M. and Geiger, B. (1991) A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. *Journal of Cell Biology*, 114: 255-262.

Mommsen, P. M. (1998) Growth and metabolism. In: *The Physiology of Fishes*, pp. 65-97. Edited by D. H. Evans. CRC Press LLC, Boca Raton, Florida.

Morgan, J. D. and Iwama, G. K. (1996) Cortisol-induced changes in oxygen consumption and ionic regulation in coastal cutthroat trout (*Oncorhynchus clarki clarki*) parr. *Fish Physiology and Biochemistry*, 15: 385-394.

Morgan, J. D. and Iwama, G. K. (1998) Salinity effects on oxygen consumption, gill Na^+,K^+-ATPase and ion regulation in juvenile coho salmon. *Journal of Fish Biology*, 53: 1110-1119.

Morgan, J. D., Sakamoto, T., Grau, E. G. and Iwama, G. K. (1997) Physiological and respiratory responses of the Mozambique tilapia (*Oreochromis mossambicus*) to salinity acclimation. *Comparative Biochemistry and Physiology*, 117A: 391-398.

Narnaware, Y. K., Kelly, S. P. and Woo, N. Y. S. (1997) Effect of injected growth hormone on phagocytosis in silver sea bream (*Sparus sarba*) adapted to hyper- and hypo-osmotic salinities. *Fish and Shellfish Immunology*, 7: 515-517.

Narnaware, Y. K., Kelly, S. P. and Woo, N. Y. S. (1998) Stimulation of macrophage phagocytosis and lymphocyte count by exogenous prolactin administration in silver sea bream (*Sparus sarba*) adapted to hyper-and hypo-osmotic salinities. *Veterinary Immunology and Immunopathology*, 61: 387-391.

Narnaware, Y. K., Kelly, S. P. and Woo, N. Y. S. (2000) Effect of salinity and ration size on macrophage phagocytosis in juvenile black sea bream (*Mylio macrocephalus*). *Journal of Applied Ichthyology*, 16: 86-88.

Negatu, Z. and Meier, A. H. (1995) In vitro incorporation of (14C)glycine into muscle protein of gulf killifish (*Fundulus grandis*) in response to insulin-like growth factor-I. *General and Comparative Endocrinology*, 98: 193-201.

Nicholl, I. D. and Quinlan, R. A. (1994) Chaperone activity of alpha-crystallins modulates intermediate filament assembly. *European Molecular Biology Organization (EMBO) Journal*, 13: 945-953.

Nishida, E., Koyasu, S., Sakai, H. and Yahara, I. (1986) Calmodulin-regulated binding of the 90-kDa heat shock protein to actin filaments. *Journal of Biological Chemistry*, 261: 16033-16036.

Norris, D. O. (1997a) Comparative aspects of the hypothalamo-hypophysial system in nonmammlian vertebrates. In: *Vertebrate Endocrinology*, pp. 165-216. Edited by D. O. Norris. Academic Press, Inc., San Diego.

Norris, D. O. (1997b) Organization of the mammalian hypothalamo-hypophysial axis. In: *Vertebrate Endocrinology*, pp. 104-164. Edited by D. O. Norris. Academic Press, Inc., San Diego.

Palleros, D. R., Reid, K. L., Shi, L., Welch, W. J. and Fink, A. L. (1993) ATP-induced protein-Hsp70 complex dissociation requires K+ but not ATP hydrolysis. *Nature*, 365: 664-666.

Pan, F., Zarate, J. M., Tremblay, G.C. and Bradley, T. M. (2000) Cloning and characterization of salmon hsp90 cDNA: upregulation by thermal and hyperosmotic stress. *Journal of Experimental Zoology*, 287: 199-212.

Pang, P. K. T., Pang, R. K. and Sawyer, W. H. (1973) Effects of environmental calcium and replacement therapy on the killifish, *Fundulus heteroclitus*, after the surgical removal of the corpuscles of Stannius. *Endocrinology*, 93:705-710.

Pickering, A. D., Pottinger, T. G., Sumpter, J. P., Carragher, J. F. and Le Bail, P. Y.

(1991) Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhynchus mykiss*. *General and Comparative Endocrinology*, 83: 86-93.

Pickart, C. M. (1997) Targeting of substrates to the 26S proteasome. *FASEB Journal*, 11: 1055-1066.

Pickart, C. M. (1999) Ubiquitin and the stress response. In: *Stress Proteins*, pp. 133-152. Edited by D. S. Latchman. Springer-Verlag, Berlin Heidelberg.

Pickford, G. E., Pang, P. K. T., Weinstein, E., Torretti, J., Hendler, E., and Epstein, F. H. (1970) The response of the hypophysectomized Cyprinodont, *Fundulus heteroclitus*, to replacement therapy with cortisol: Effects on blood serum and sodium-potassium activated adenosine triphosphatase in the gills, kidney, and intestinal mucosa. *General and Comparative Endocrinology*, 14: 524-534.

Planas, J. V., Swanson, P., Rand-Weaver, M. and Dickhoff, W. W. (1992) Somatolactin stimulates in vitro gonadal steroidogenesis in coho salmon, *Oncorhynchus kisutch*. *General and Comparative Endocrinology*, 87: 1-5.

Pottinger, T. G., Prunet, P. and Pickering, A. D. (1992) The effects of confinement stress on circulating prolactin levels in rainbow trout (*Oncorhynchus mykiss*) in fresh water. *General and Comparative Endocrinology*, 88: 454-460.

Prunet, P., Boeuf, G., Bolton, J. P. and Young, G. (1989) Smoltification and seawater

adaptation in Atlantic salmon (*Salmo salar*): plasma prolactin, growth hormone, and thyroid hormones. *General and Comparative Endocrinology*, 74: 355-364.

Rabindran, S. K., Haroun, R. I., Clos, J., Wisniewski, J. and Wu, C. (1993) Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. *Science*, 259: 230-234.

Rabindran, S. K., Wisniewski, J., Li, L., Li, G. C. and Wu, C. (1994) Interaction between heat shock factor and hsp70 is insufficient to suppress induction of DNA-binding activity in vivo. *Molecular and Cellular Biology*, 14: 6552-6560.

Rand-Weaver, M., Baker, B. J. and Kawauchi, H. (1991) Cellular localization of somatolactin in the pars intermedia of some teleost fishes. *Cell and Tissue Research*, 263: 207-216.

Rand-Weaver, M., Noso, T. and Kawauchi, H. (1991) The complete amino acid sequences of two variants of growth hormone from Atlantic cod (*Gadus morhua*). *General and Comparative Endocrinology*, 81: 39-50.

Rand-Weaver, M., Pottinger, T. G. and Sumpter, J. P. (1993) Plasma somatolactin concentrations in salmonid fish are elevated by stress. *Journal of Endocrinology*, 138: 509-515.

Rao, P. V., Huang, Q. L., Horwitz, J. and Zigler, J. S. Jr. (1995) Evidence that alpha-crystallin prevents non-specific protein aggregation in the intact eye lens.

Biochimica et Biophysica Acta, 1245: 439-447.

Ryan, J.A. and Hightower, L. E. (1996) Stress proteins as molecular biomarmkers for environmental toxicology. In: *Stress-Inducible Cellular Responses*, pp. 411-424. Edited by U. Feige, R. I. Morimoto, I. Yahara and B. S. Polla. Birkhäuser Verlag Basel, Switzerland.

Sakai, M., Kobayashi, M. and Kawauchi, H. (1995) *In vitro* activation of fish phagocytic cells by GH, prolactin and somatolactin. *Journal of Endocrinology*, 151: 113-118.

Sakal, E., Elberg, G. and Gertler, A. (1997) Direct evidence that lactogenic hormones induce homodimerization of membrane-anchored prolactin receptor in intact Nb-2-11C rat lymphoma cells. *FEBS Letters*, 410: 289-292.

Sanchez, Y., Taulien, J., Borkovich, K. A. and Lindquist, S. (1992) Hsp104 is required for tolerance to many forms of stress. *(European Molecular Biology Organization (EMBO) Journal*, 11: 2357-2364.

Sathiyaa, R., Campell, T. and Vijayan, M. M. (2001) Cortisol modulates HSP90 mRNA expression in primary cultures of trout hepatocytes. *Comparative Biochemistry and Physiology*, 129B: 679-685.

Scheibel, T. and Buchner, J. (1998) The Hsp90 complex - a super-chaperone machine as a novel drug target. *Biochemical Pharmacology*, 56: 675-682.

Sharma, H. S., Nyberg, F., Gordh, T., Alm, P. and Westman, J. (2000) Neurotrophic factors influence upregulation of constitutive isoform of heme oxygenase and cellular stress response in the spinal cord following trauma: An experimental study using immunohistochemistry in the rat. *Amino Acids (Vienna)*, 19: 351-361.

Shrimpton, J. M., Devlin, R. H., McLean, E., Byatt, J. C., Donaldson, E. M. and Randall, D. J. (1995) Increases in gill corticosteroid receptor abundance and saltwater tolerance in juvenile coho salmon (*Oncorhynchus kisutch*) treated with growth hormone and placental lactogen. *General and Comparative Endocrinology*, 98: 1-15.

Singh, H., Griffith, R .W., Takahashi, A., Kawauchi, H., Thomas, P. and Stegeman, J. J. (1988) Regulation of gonadal steroidogenesis in *Fundulus heteroclitus* by recombinant growth hormone and purified salmon prolactin, *General and Comparative Endocrinology*, 72: 144-153.

Smith, T. R., Tremblay, G. C. and Bradley, T. M. (1999) Hsp70 and a 54 kDa protein (Osp54) are induced in salmon (*Salmo salar*) in response to hyperosmotic stress. *Journal of Experimental Zoology*, 284: 286-298.

Soltys, B. J. and Gupta, R. S. (1999) Mitochondrial molecular chaperones hsp60 and mhsp70: are their roles restricted to mitochondria? In: *Stress Proteins*, pp. 69-100. Edited by D. S. Latchman. Springer-Verlag, Berlin Heidelberg.

Sonntag, W. E. (1996) An overview of the biological actions and neuroendocrine regulation of growth hormone. In: *Handbook of Endocrinology*, pp. 171-202. Edited by

G. H. Gass and H. M. Kaplan. CRC Press LLC, Boca Raton, Florida.

Specker, J. L., Kishida, M., Huang, L., King, D. S., Nagahama, Y., Ueda, H. and Anderson, T. R. (1993) Immunocytochemical and immunogold localization of two prolactin isoforms in the same pituitary cells and in the same granules in the tilapia (*Oreochromis mossambicus*). *General and Comparative endocrinology*, 89: 28-38.

Swanson, C. (1998) Interactive effects of salinity on metabolic rate, activity, growth and osmoregulation in the euryhaline milkfish (*Chanos chanos*). *Journal of Experimental Biology*, 201: 3355-3366.

Takayama, Y., Ono, M., Rand-Weaver, M. and Kawauchi, H. (1991) Greater conservation of somatolactin, a presumed pituitary hormone of the growth hormone/prolactin family, than of growth hormone in teleost fish. *General and Comparative Endocrinology*, 83: 366-374.

Udelsman, R., Blake, M. J., Stagg, C. A. and Holbrook, N. J. (1994) Endocrine control of stress-induced heat shock protein 70 expression in vivo. *Surgery (St Louis)*, 115: 611-616.

Ungermann, C., Guiard, B., Neupert, W. and Cyr, D. M. (1996) The and Hsp70/Mim44-dependent reaction cycle driving early steps of protein import into mitochondria. *European Molecular Biology Organization (EMBO) Journal*, 15: 735-744.

Ungermann, C., Neupert, W. and Cyr, D. M. (1994) The role of Hsp70 in conferring unidirectionality on protein translocation into mitochondria. *Science*, 266: 1250-1253.

Veillette, P. A., Sundell, K. and Specker, J. L. (1995) Cortisol mediates the increase in intestinal fluid absorption in Atlantic salmon during parr-smolt transformation. *General and Comparative Endocrinology*, 97: 250-258.

Vestling, M., Murphy, C., Fenselau, C. and Chen, T. T. (1991) Disulfide bonds in native and recombinant fish growth hormones. *Molecular Marine Biology and Biotechnology*, 1: 73-77.

Viitanen, P. V., Lorimer, G. H. Seetharam, R., Gupta, R. S., Oppenheim, J., Thomas, J. O. and Cowan, N. J. (1992) Mammalian mitochondrial chaperonin 60 functions as a single toroidal ring. *Journal of Biological Chemistry*, 267: 695-698.

Vijayan, M. M., Pereira, C., Forsythe, R. B., Kennedy, C. J. and Iwama, G.K. (1997a) Handling stress does not affect the expression of hepatic heat shock protein 70 and conjugation enzymes in rainbow trout treated with beta-naphthoflavone. *Life Sciences*, 61: 117-127.

Vijayan, M. M., Pereira, C., Grau, E. G. and Iwama, G. K. (1997b) Metabolic responses associated with confinement stress in tilapia: the role of cortisol. *Comparative Biochemistry and Physiology*, 116C: 89-95.

Vijayan, M. M., Pereira, C., Kruzynski, G. and Iwama, G. K. (1998) Sublethal

concentrations of contaminant induce the expression of hepatic heat shock protein 70 in two salmonids. *Aquatic Toxicology*, 40: 101-108.

Wachter, C., Schatz, G. and Glick, B. S. (1994) Protein import into mitochondria: The requirement for external ATP is precursor-specific whereas intramitochondrial ATP is universally needed for translocation into the matrix. *Molecular Biology of the Cell*, 5: 465-474.

Welch, W. J., Eggers, D. K., Hansen, W. J. and Nagata, H. (1998) Chaperone interactions with nascent and newly synthesized proteins. In: *Molecular Chaperones in the Life Cycle of Proteins*, pp. 71-93. Edited by A. L. Fink and Y. Goto. Marcel Dekker, Inc., New York.

Wendelaar Bonga, S. E. (1978) The effects of changes in external sodium, calcium, and magnesium concentrations on prolactin cells, skin, and plasma electrolytes of *Gasterosteus aculeatus*. *General and Comparative Endocrinology*, 34: 265-275.

Wendelaar Bonga, S. E. (1997) The stress response in fish. *Physiological Reviews*, 77: 591-625.

Wiech, H., Buchner, J., Zimmermann, R. and Jakob, U. (1992) Hsp90 chaperones protein folding in vitro. *Nature*, 358: 169-170.

Wong, A. O. L., Chang, J. P. and Peter, R. E. (1992) Dopamine stimulates growth hormone release from the pituitary of goldfish, *Carassius auratus*, through the

Woo, N. Y. S., Deane, E. E. and Li. J. (1999) Heat shock protein (HSP) expression in response to environmental stress in cultured sea bream. Mini-Symposium on Aquacultural Biotechnology, New Asia College, The Chinese University of Hong Kong. 10-11 December 1999.

Xu, Y. and Lindquist, S. (1993) Heat-shock protein hsp90 governs the activity of pp60-v-src kinase. *Proceedings of the National Academy of Sciences of the United States of America*, 90: 7074-7078.

Yada, T., Hirano, T. and Grau, E. G. (1994) Changes in plasma levels of the two prolactins and growth hormone during adaptation to different salinities in the euryhaline tilapia, *Oreochromis mossambicus*. *General and Comparative Endocrinology*, 93: 214-223.

Yamashita, M., Ojima, N. and Sakamoto, T. (1996) Induction of proteins in response to cold acclimation of rainbow trout cells. *FEBS Letters*, 382: 261-264.

Young, G., Björnsson, B. T., Prunet, P., Lin, R. J. and Bern, H. A. (1989) Smoltification and seawater adaptation in coho salmon (*Oncorhynchus kisutch*): plasma prolactin, growth hormone, thyroid hormones, and cortisol. *General and Comparative Endocrinology*, 74: 335-345.

Zar, J. H. (1984) *Biostatistical Analysis*, (2nd edition). Upper Saddler River, New Jersey. Prentice Hall Inc.

Zhu, Y. and Thomas, P. (1995) Red Drum somatolactin: development of a homologous radioimmunoassay and plasma levels after exposure to stressors or various backgrounds. *General and Comparative Endocrinology*, 99: 275-288.

Zhu, Y. and Thomas, P. (1996) Elevations of somatolactin in plasma and pituitaries and increased alpha-MSH cell activity in red drum exposed to black background and decreased illumination. *General and Comparative Endocrinology*, 101: 21-31.